Universidade de Aveiro Departamento de Biologia
Ano 2015

Jéssica Raquel Cortez Marques

À procura do perfil molecular característico da Perturbação de Hiperatividade e Défice de Atenção e da Dislexia.

In search of the molecular profile characteristic of Attention Deficit Hyperactivity Disorder of and Dyslexia

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Professora Doutora Marlene Maria Tourais de Barros, Professora Associada com Agregação do Departamento de Ciências da Saúde da Universidade Católica Portuguesa e coorientação da Professora Doutora Ana Cristina Esteves, Professora Auxiliar convidada do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave

Saliva, perturbação de hiperatividade e défice de atenção, dislexia, diagnóstico salivar, biomarcadores inflamatórios, inflamação, tecnologia multiplex

resumo

A Perturbação de Hiperatividade e Défice de atenção e a Dislexia são doenças do neuro-desenvolvimento com uma prevalência muito elevada nas crianças e que frequentemente persistem na adolescência e no adulto. Com um diagnóstico difícil, baseado em escalas nas quais se quantificam os sinais e sintomas, um método de diagnóstico fiável, não invasivo e adequado a crianças é apropriado. Para isso, um diagnóstico utilizando amostras de saliva com recurso a biomarcadores salivares é o ideal para a população em estudo. O estabelecimento do SOP com procedimentos de recolha, processamento e armazenamento são essenciais para o uso da saliva.

Além disso, e uma vez que parece haver relação entre doenças cognitivas e inflamação, a avaliação do perfil de proteínas e quantificação de citocinas relacionadas com a inflamação são um ponto de partida para entendermos estas patologias. A avaliação de duas citocinas CCL3 e CCL13, presentes em inflamação, foi realizada com recurso à tecnologia multiplex. Os níveis destas duas citocinas encontram-se aumentados em indivíduos multi-comprometidos o que nos leva a inferir que a neuroinflamação está presente nestas patologias. Estudos futuros devem ser feitos no sentido de validar biomarcadores inflamatórios nestas patologias.

keywords

Saliva, Attention Deficit Hyperactivity Disorder, dyslexia, salivary diagnosis, inflammatory biomarkers, inflammation, multiplex technology

abstract

The Attention Deficit Hyperactivity Disorder and Dyslexia are neurodevelopmental diseases with a very high prevalence in children which often persist into adolescence and adulthood. With a difficult diagnosis, based on scales quantifying the signs and symptoms, a method of reliable, non-invasive diagnosis, suitable for children is urgent. A diagnosis using saliva samples quantifying salivary biomarkers is ideal for this study population. The establishment of the standard operating procedure with procedures for collecting, processing and storage of samples is essential to use saliva.
Furthermore, since there appears to be a relationship between cognitive diseases and inflammation, the evaluation of the protein profile and quantification of cytokines related to inflammation are a starting point to understand the pathology. The evaluation of the chemokine (c-c motif) ligand 13 (CCL13) and of the chemokine (c-c motif) ligand 3 (CCL3), present in inflammation, was done using multiplex technology. The levels of these two chemokines are increased in multicompromised individuals which leads us to infer that neuroinflammation is present in these pathologies. Future studies should be done to validate inflammatory biomarkers in these diseases.

Communications

Panel Communications

Marques, J; Esteves, E; Fernandes, M; Rosa, N; Correia, M; Barros, M “Quality control of saliva samples for SalivaTec”. I Jornadas Científicas da Primavera, Universidade Católica Portuguesa, Viseu, March 20, 2015

Marques, J; Rosa, N; Correia, M; C, Ribeiro; Barros, M “In search of the molecular profile characteristic of Attention Deficit Hyperactivity Disorder (ADHD) and Dyslexia”. I Jornadas Científicas da Primavera, Universidade Católica Portuguesa, Viseu, March 20, 2015

Marques, J; Rosa, N; Correia, M; C, Ribeiro; Barros, M “In search of the molecular profile characteristic of Attention Deficit Hyperactivity Disorder (ADHD) and Dyslexia”. Dislexia e Hiperatividade em debate, Universidade Católica Portuguesa, Viseu, May 23, 2015

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Abbreviations

AD: Alzheimer's disease

ADHD: Attention deficit hyperactivity disorder

AKT: Protein kinase B

CCL: Chemokine (C-C motif) ligand

Ig: Immunoglobulin

IL: Interleukin

IMM: Instituto de Medicina Molecular

JAK/STAT: Janus kinase/signal transducers and activators of transcription

MHC: Major histocompatibility complex

MS: Mass Spectrometry

mTor: Mammalian target of rapamycin

MW: Molecular Weight

PD: Parkinson's disease

PKA: Protein kinase A

SOP: Standard operating procedure

TLR: Toll-like receptors

TNF: Tumor necrosis factor

1. Introduction

1.1 Attention deficit hyperactivity disorder

1.1.1 Characterization of attention deficit hyperactivity disorder

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder defined by a persistent standard of lack of attention and/or hyperactivity-impulsivity that interferes with the function of development [1]. Typically, it manifests itself early in development, before the child starts school, and is characterized by developmental deficits that affect the operation at personal, social, academic and occupational levels. It is one of the most common childhood neurobehavioral disorders that frequently persist into adolescence and adulthood. In many cases, even with intervention, the difficulties persist into adolescence and adulthood with deficits that include specific limitations on learning, executive functions, intelligence and social skills [2, 3]. The significant functional impairment, poorer quality of life, legal problems, grade retention, lower standardized achievement scores and grade point averages, failure to graduate high school, increased rates of substance abuse and persistent neuropsychological impairments are frequently associated with ADHD and represent increased costs to the individual and the society [4]. The ADHD occurs in about ~5 to 8% of children and 2.5% of adults. It is more prevalent in men, in a ratio of two to one, but in adolescence this discrepancy is attenuated, with both genders presenting a similar prevalence [5].

1.1.2 Neurophysiology of ADHD

The cardinal features of ADHD are problems in the domains of the attention, impulse and motor control [6]. The brain region involved in the control of action and attention include the prefrontal cortex (particularly the dorsolateral cortex), striatum (composed by two structures, the caudate and the putamen) and the cerebellum (particularly the vermis), regions that are interconnected [6] **(Figure 1)**.

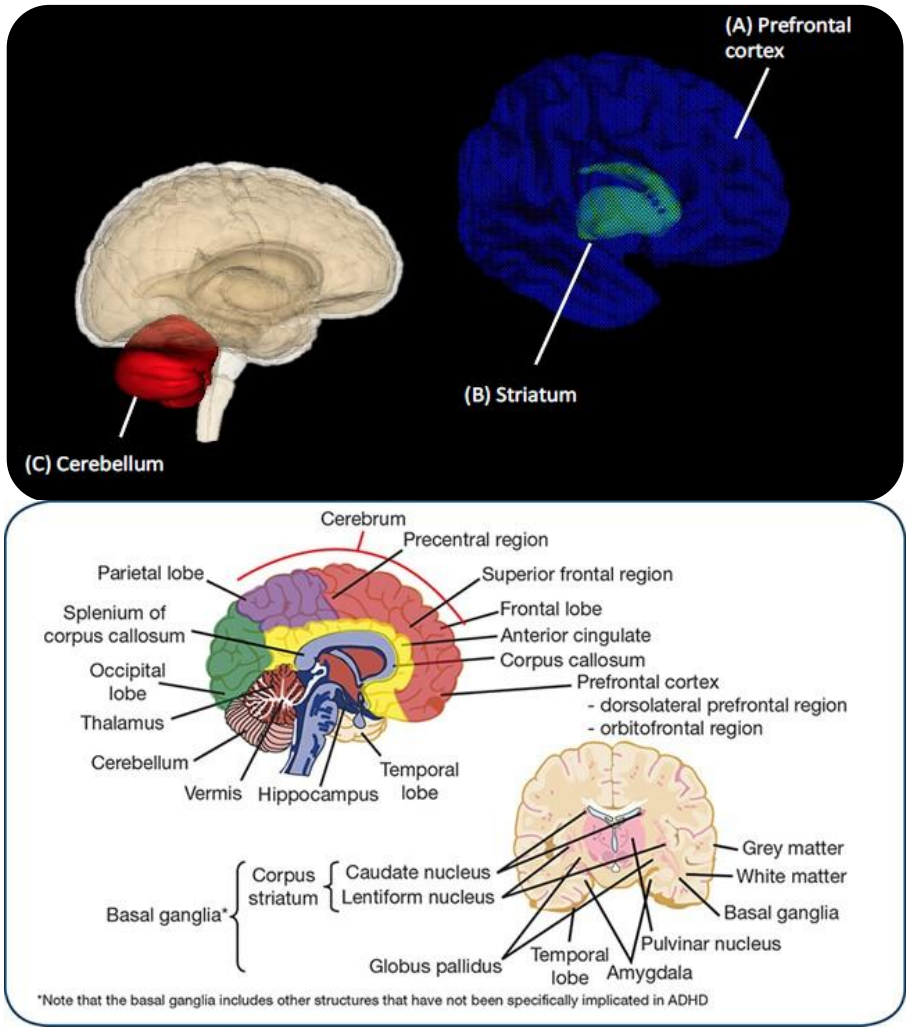


Figure 1 - The key components involved in the control of action and attention centers in the prefrontal cortex (A), the striatum (B), and the cerebellum (C). Adapted by [6] and from [7].

Individuals with ADHD present subtle differences in brain structure, like a decreased in volume of the cerebellum [8, 9], and striatum [6]. Measures of brain cortical and subcortical volumes show that the cerebrum as a whole is smaller in children and adolescents with ADHD [10] **(Figure 2)**.

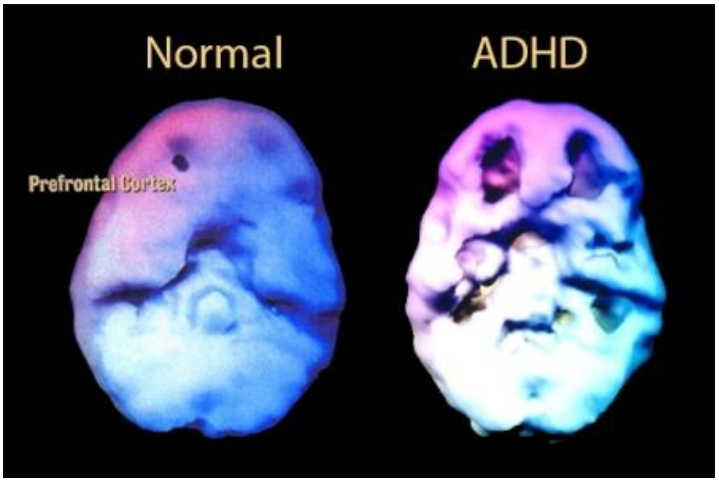


Figure 2 – Comparison between normal brain and ADHD brain. From [11].

1.1.3 Etiology of the Disease

Family histories of ADHD, preterm birth, smoking or drinking during pregnancy are risk factors for developing ADHD [12-14]. Psychological adversity and high levels of family conflicts were also associated with the disease [15]. *In utero*, several factors that include the maternal stress during pregnancy [16], prenatal exposure to tobacco, alcohol and other drugs and environmental toxins [17, 18], pregnancy/birth complications [17], intrauterine growth retardation [19] and low birth weight/prematurity have been associated with ADHD.

Neonatal axonia and seizures, brain injury [17] and exposure to lead [20] are early postnatal environmental factors that influence ADHD.

1.1.4 Diagnosis of ADHD

The diagnosis of this pathology is based on clinical observation, but some rating scales, such the *Conners Rating Scale and the Strengths and Difficulties Questionnaire*, completed by parents and teachers, can be useful to detect and quantify the signs and symptoms [21, 22]. The diagnostic is not easy due to the comorbidity with other pathologies like autism spectrum disorder, depression, anxiety, bipolar, antisocial and mood disorders [23-25]. An early detection is

required for an early intervention, either pharmacological, behavioral or pedagogical [5]. Since this pathology manifests itself in children, non-invasive, comfortable and pain-free methods to help to diagnose this pathology are required.

1.1.5 Treatment for ADHD

The treatments include pharmacological and/or behavior therapy. The pharmacological therapy is considered the first-line treatment for patients with severe ADHD and impairment [12]. Medications for these patients (approved by the Food and Drug Administration) include stimulant and nonstimulant medications. In terms of stimulant medication, there are two categories: amphetamines and methylphenidates. Data suggest that amphetamines may be moderately more effective than methylphenidates [26].

Methylphenidate is the most common used and is rapidly absorbed orally. It starts to act 20-30 minutes after ingestion and is eliminated quickly, wherein the effect is maintained only during 3 or 4 hours [27]. In terms of nonstimulant medication the atomoxetine is the most common. Atomoxetine blocks the norepinephrine transporter and increases levels of both noradrenalin and dopamine in the prefrontal cortex [28]. The principal adverse effects of taking these drugs are decreased appetite, headache, dry mouth, insomnia, irritability and dizziness [29].

1.1.6. ADHD and metabolism

Individuals with ADHD have a deficiency of micronutrients, such as folate, vitamin B6, zinc, carnitine, serine, glutamine, choline and antioxidants **(Figure 3)**.

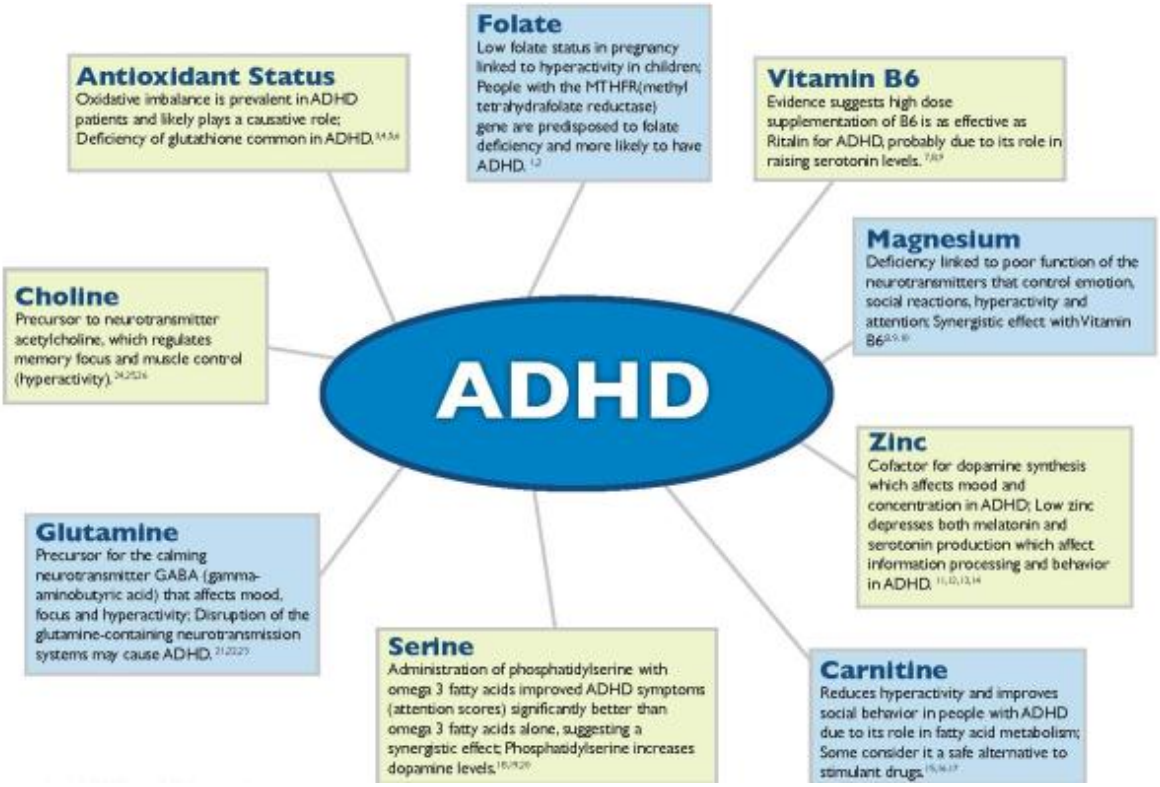


Figure 3 - Nutrition correlation status in ADHD. From [30].

In terms of antioxidant status, individuals with ADHD have a prevalent oxidative imbalance and glutathione deficiency is also common. A study correlates the low folate status in pregnancy with hyperactivity in children [31]. People with methyl tetrahydrafolate reductase gene are predisposed to folate deficiency and are more expected to have ADHD [32].

Evidences suggest that as Ritalin, high doses of vitamin B6 are effective for patients with ADHD, possibly due to its role in raising serotonin levels [33]. This vitamin also has a synergistic effect with magnesium. In terms of nutrients, deficiency of magnesium leads to poor function of neurotransmitters that control emotion, social reactions and attention which are involved in ADHD [34]. Zinc is an essential nutrient important in immune system function. Low levels of zinc depress melatonin and serotonin production which affect information processing and behavior in ADHD [35]. Studies have shown that zinc levels are lower than normal in the majority of children with ADHD [36]. Carnitine reduces hyperactivity and

improves social behavior in individuals with ADHD due to its role in fatty acid metabolism. Carnitine is considered by many a safe alternative to stimulant drugs [37]. Administration of phosphatidylserine with omega 3 fatty acids improved ADHD symptoms suggesting a synergistic effect [38]. Also, phosphatidylserine increases dopamine levels. Glutamine that is the precursor for the neurotransmitter gamma-aminobutyric acid affects mood, focus and hyperactivity [39]. Disruption of the glutamine-containing neurotransmission systems may cause ADHD [39]. Choline a precursor of acetylcholine regulates memory, focus and muscle control, areas which are related with hyperactivity [40].

1.1.7 Physiopathology of the disease

Several central nervous system abnormalities in ADHD patients confirm the neurobiological basis of the disorder. Findings show that other neurodevelopmental disorders like autism, schizophrenia, and epilepsy share genetic variants with ADHD [41]. The brain sub regions including frontal and parietal cortexes, basal ganglia, cerebellum, hippocampus, and corpus callosum have been involved in the functional networks related to ADHD [42] **(Figure 4)**.

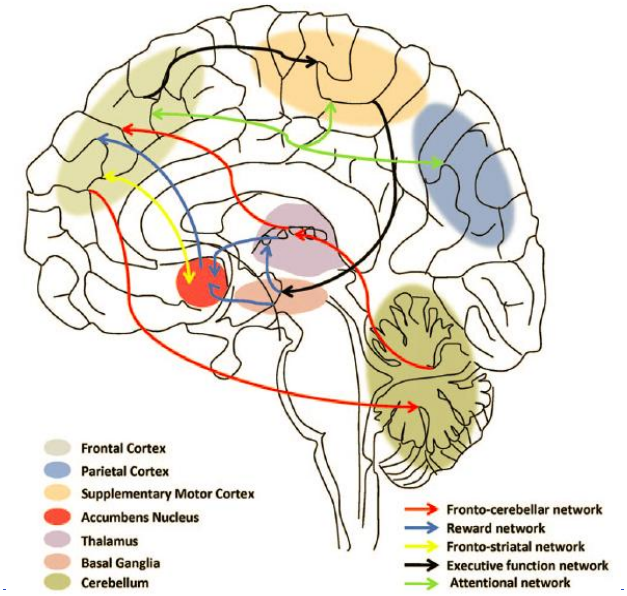


Figure 4- Schematic representation of functional circuits involved in the pathophysiology of ADHD. Here the attention network (green), the fronto-striatal network (yellow), the executive function network (black), the fronto-cerebellar network (red), and the reward network (blue) are summarized. From [28]

ADHD is related to a dysfunction of the circuits at the nervous system level, reducing the availability of neurotransmitters like dopamine and noradrenalin [43], and these deficits may underlie core symptoms of lack of attention [44] and impulsivity [45].

1.1.7.1 Membrane trafficking and protein kinases involved in ADHD

There is genetic evidence linking the components of membrane trafficking of intracellular vesicles to a variety of neurological conditions including autism and ADHD. Dopamine transporter-mediated re-uptake system controls the intensity and duration of dopamine actions at synaptic receptors, which provides modulatory influences over attention and behavior. Dopamine signaling is a crucial risk factor for ADHD, and dopamine transporter may be involved in the dopaminergic dysfunction associated with ADHD [46].

The intra-cellular signaling of dopamine transporter may happen through the protein kinase A (PKA) and protein kinase B (AKT) pathways. AKT is a central player in signal transduction activated in response to numerous growth factors and is thought to contribute to many important cellular functions, including cell growth, apoptosis, nutrient metabolism, and modulating the activity of several transcription factors [47] **(Figure 5)**.

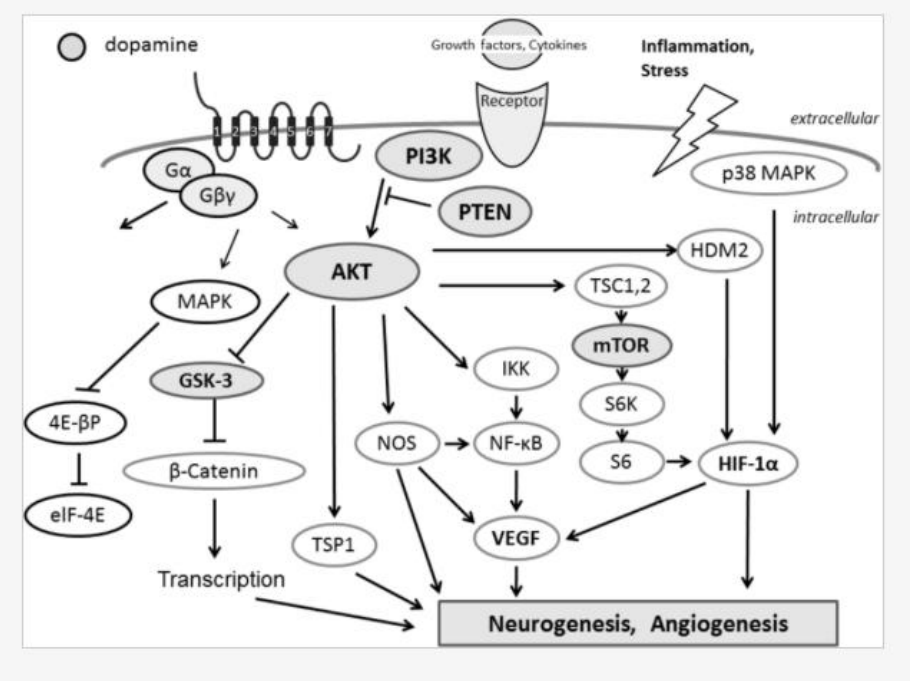


Figure 5- Phosphoinositide 3-kinase/ Protein kinase B/ Phosphatase and tensin homolog signaling. PI3K (Phosphoinositide 3-kinase); PTEN (Phosphatase and tensin homolog); AKT (Protein kinase B); mTor (mammalian target of rapamycin); From [48].

In addition, a crosstalk between PKA and mammalian target of rapamycin (mTOR) pathway in apoptosis resistance signaling has been reported [86]. mTOR is a conserved serine/threonine kinase within the cells that controls protein synthesis and cell growth and is involved in neurological disorders including ADHD and autism [49]. Two mTOR complexes (1 and 2) are also involved in ADHD and autism. Deregulation in mTOR signaling, namely the loss of mTORC2 signaling lead to disrupt normal brain development and its function. mTORC2 has a role in neuronal size control and its deregulation affects function, size and connectivity of neurons [50].

The critical role of protein kinases in brain development has been investigated for decades. A study done in rats shows that PKA inhibition within the medial prefrontal cortex produces inattention and hyperactivity, and might modulate human attention disorders. Considering the implication of protein kinases in ADHD, it is important to understand the effect of each one in inflammatory responses in the brain. PKA controls the expression of some key cytokines like interleukin (IL)-6 an interleukin that acts as a pro-inflammatory and anti-inflammatory cytokine.

Both activation and inhibition of the two kinases if they work one-sidely, may not contribute to the improvement of neuronal disorders [48] **(Figure 6)**.

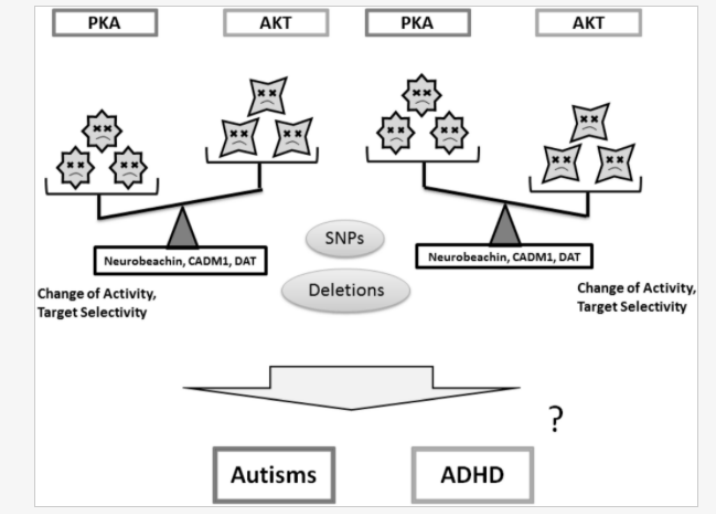


Figure 6- Scheme of protein kinases PKA and AKT modulation in the pathogenesis of autisms and ADHD. Star faces represent an image of the individual kinase activities. Sad faces mean unbalance of kinase activity. PKA (Protein kinase A); AKT (Protein kinase B); SNPs (Single Nucleotide Polymorphisms); CADM1 (cell adhesion molecule 1); DAT (Dopamine Transporter); ADHD (Attention Deficit Hyperactivity disorder). In [48].

The alteration of the functions in neurobeachin, cell adhesion molecule 1 and dopamine transporter with genetic deletion and/or single nucleotide polymorphisms may change the activity or selectivity of these kinases to substrates, which in turn may cause the psychological disorders [48].

The balance between PKA and AKT kinases may be essential for their function. Several food and/or dietary components can contribute to the balance via the modulation of kinase activities [48]. These findings might be translated into new dietary managements for the treatment of diseases like autisms and ADHD in the future [48]. ADHD has been suggested to be related to a deficiency of the omega-3 long-chain polyunsaturated fatty acids [51]. ω-*3* long-chain polyunsaturated fatty acids low levels in blood have been reported in children with ADHD and related to learning difficulties, suggesting benefits from dietary supplementation [52].

Recent studies highlight the contribution of the intestinal flora to psychiatric diseases [53, 54]. The brain and the gut form an axis of two-way communication [55], and the alteration of this axis can lead to psychiatric disorders such as perturbations of the autism spectra [56], mood disorders, anxiety and depression [55, 57].

1.1.8 Link between ADHD and Inflammation

Increasing evidence indicates that brain inflammation is important in the pathogenesis of neuropsychiatric disorders [58-60]. Brain inflammation is a commonly complex process involving several types of cells, including microglia, monocytes, neutrophils, astrocytes and neurons [61]. Until recently, it was thought that the microglia, resident brain macrophages, was the only cell mediating brain inflammation. However, recently it was shown that neutrophils and monocytes infiltrate the injured brain and contribute to inflammation. Neurons and astrocytes also participate in brain inflammation [62]. The astrocytes are responsible for producing anti-inflammatory factors [63, 64] and chemokines that recruit monocytes [65]. Neurons regulate the inflammation positively and negatively [66]. Microglia, in response to injury, isolate damaged areas and prevent propagation of disrupted injury sites and express chemokines and growth factors that support survival of surrounding neurons. Then, neutrophils infiltrate in case of risk of infection. Thereafter, monocytes infiltrate, helping to repair brain damage [61].

The activation of the innate immune signaling pathways in microglial cells occurs in response to infectious organisms, during brain injury and chronic disease [67]. Similarly to macrophages, microglial cells express toll-like receptors (TLRs), respond to TLR ligands and produce pro-inflammatory mediators [68, 69]. Microglial cells can be activated during systemic infections without the integrity of the blood-brain barrier being compromised. In regions where there is no blood-brain barrier, the response to circulating pathogens is similar to that in most systemic organs [67].

Microglia comprises a rather large cell population and distinct stimuli trigger distinct responses **(Figure 7)**.

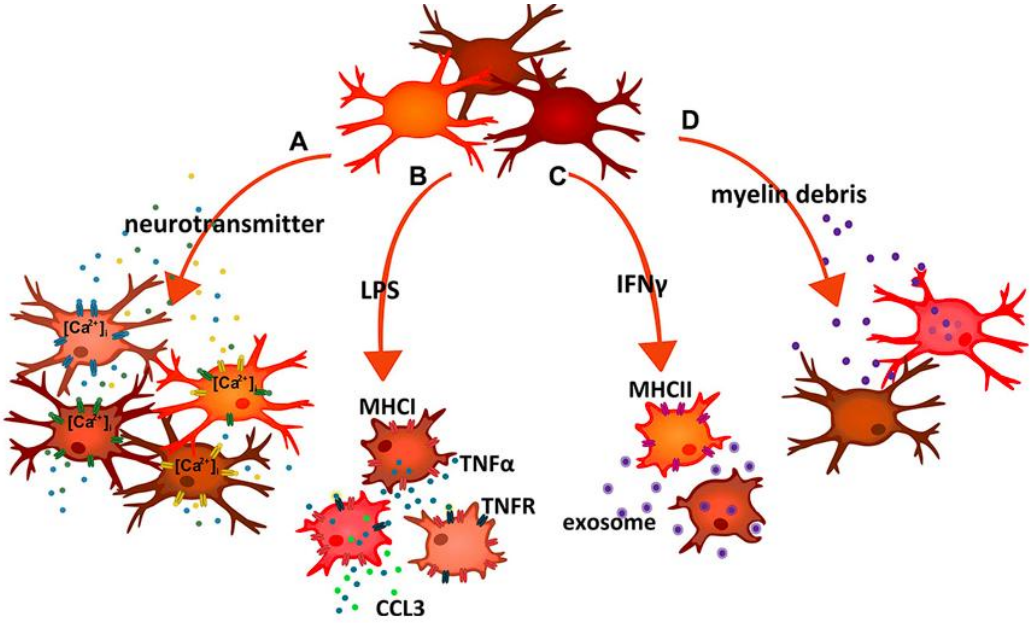


Figure 7- Microglia response heterogeneity. (A) The stimulation of microglia by neurotransmitters and neurohormones trigger calcium (Ca^{2+}) signals. (B) Exposure to lipopolysaccharides (LPS) induce a population expression of major histocompatibility complex 1 (MHCI) molecules. All microglia cells express TLR4 but only some subsets produce TNF-α and/or CCL3. (C) The stimulation by interferon gamma (IFN-γ) gamma causes the expression of major histocompatibility complex II (MHCII) in some cells. (D) Exposure of microglia to myelin debris results in phagocytic uptake of the material. In [70].

The microglial diversity when stimulated by neurotransmitters and hormones **(Figure 7A)** triggers calcium signals. Exposure to lipopolysaccharides (LPS) **(Figure 7B)** induces a population expression of major histocompatibility complex 1 (MHC class I) molecules. Almost all cells showed TLR4-mediated induction of MHCI. Stimulation of mouse microglial TLR4 trigger the panpopulation upregulation of MHC class I, required for antigen presentation to cytotoxic T cells. This TLR4 activation leads to the release of TNF-α. At a single cell level, only a subpopulation can carries this activity, the demarcation of the subpopulation gets sharper with increased postnatal age. That includes chemokine (C-C motif) ligand

(CCL) 3 as a T cell-attracting chemokines. Concluding, all microglia cells express TLR4 but only some subsets produce TNF-α and/or CCL3.

The stimulation by Interferon gamma **(Figure 7C)** causes the expression of major histocompatibility complex II (MHC class II) structures for antigen presentation in some cells. Exposure of microglia to myelin debris **(Figure 7D)** results in the phagocytic uptake of the materials.
Microglia response heterogeneity may itself be part of the pathogenic process or rather an attempt to contain its aggravation.

Depression, anxiety, sleep disorders and dyslexia are often associated with ADHD. Evidences suggest that hyperactive individuals are commonly anxious and depressive, and these two conditions have been suggested to be associated with systemic inflammation up-regulation [71]. There is an association between anxiety characteristics and inflammatory biomarkers such C reactive protein, interleukin-6 (IL-6) and cortisol. [72, 73] The levels of c reactive protein are increased only in anxious and depressive men [72]. In anxious individuals, the levels of morning cortisol are lower and the levels of the pro-inflammatory IL-6 are higher [73].

There is evidence indicating that inflammation underlies other neurological pathologies, such as dementia [74], Alzheimer's disease (AD) [75], depression [76] and psychological stress [77]. However, there is a lack of molecular data. Evidences indicate that systemic infection has an impact on the inflammatory diseases of the central nervous system which influence the disease process and the neurological function [78]. Systemic infections increase cytokines synthesis [79] and these circulating cytokines and other inflammatory mediators can affect the brain by several routes [78]. Circulating cytokines like IL1β, IL-6 and TNF-α cannot penetrate the blood brain barrier [80], thus they communicate with the brain centers through the cerebral endothelium and act on brain through the circumventricular organs [80] and the vagus nerve to effect local cytokine and prostaglandin synthesis and produce sickness behaviours.

1.1.9 Chemokines

Microglia and astrocytes are prominent sources of chemokines within the central nervous system, but some neurons also produce chemokines. Chemokines are important signaling molecules in the central nervous system. They belong to a family of small proteins identified for their role in immune system as inflammatory and chemoattractive factors.

There are two types of chemokines: the inflammatory chemokines that control the recruitment of leucocytes during infection [81], the interaction with members of the G protein-coupled receptor [82], inflammation, tissue injury and play a role in T cell activation and development [83, 84] and the homeostatic chemokines that navigate leucocytes during hematopoiesis in the bone marrow and thymus [85].

1.1.9.1 Chemokine CCL3

CCL3 is a 7.8 kDa protein that is up regulated in the hippocampus of temporal lobe epilepsy patients [86]. The increased levels of CCL3 occur during epileptogenesis in animal models of temporal lobe epilepsy which suggest a role for CCL3 in this condition [87]. CCL3 has also been implicated in head injury [88], ischemia [89], AD [90] and HIV infection [91].

Functional properties of central nervous system neurons could be altered by CCL3 if subjected to prolonged exposure of this chemokine in the central nervous system, as occurs during CNS neuroinflammation [92].

1.1.9.2 Chemokine CCL13

CCL13 is a member of a distinct, structurally related subclass of chemoattractants chemokines mainly involved in recruitment of eosinophils, basophils monocytes and T lymphocytes to inflammatory sites [93].

This chemokine is up-regulated at sites of inflammation and may play a major role in the pathophysiological mechanisms of allergic disorders such asthma [94] and atopic dermatitis [95], which are considered to be Th2 dominant diseases.

Expression of CCL13 protein is greater in the sputum, epithelium, submucosal inflammatory cells and bronchoalveolar lavage fluid of asthmatics individuals [94].

CCL13 is a chemokine that has been detected and quantified in blood [93] and it is also highly expressed in cartilage from patients with rheumatoid arthritis [96].

1.2. Characterization of dyslexia

Reading disorders like dyslexia and attention deficits often co-occur [97]. Dyslexia is a neurodevelopmental disorder characterized by slow and inaccurate word recognition and spelling [98]. Individuals with this pathology have difficulties with decoding regardless of adequate instruction, intelligence and intact sensory abilities and comprehension [98]. Dyslexia can result from brain damage (acquired dyslexia) or be present before reading acquisition (developmental dyslexia) [97]. The definition of dyslexia assumes that the disorder is neurobiological in origin with strong evidence for heritability, but environmental factors shape the risk for the disease [99]. The prevalence of dyslexia in school-age children ranges between 6% and 17% [100]. The signs and symptoms of this pathology are caused by a deficit in specific language skills responsible for processing phonological information [101]. The diagnosis is done after the individual is exposed to formal literacy instruction. [102, 103]. Dyslexic males have higher rates of comorbid disorders such as ADHD and come to clinical attention more often than females, which can indicate the risk for later reading problems [104, 105]. This pathology can be prevented in children with an early intervention [99].

Detecting reading disorders like dyslexia as early as possible may help individuals to overcome reading and learning problems. A study identifies a specific gene, DYX1C1, as a candidate susceptibility gene for developmental dyslexia. This gene encodes a nuclear tetratricopeptide repeat domain protein dynamically regulated in brain. The DYX1C1 protein appears to be rapidly up-regulated and translocated after brain ischemia [106].

1.3 Saliva as a diagnostic fluid

Human saliva is a complex body fluid composed by an exocrine contribution from the major and minor salivary glands and by non-exocrine components that comprise microorganisms, desquamated oral epithelial cells and leukocytes [107, 108] . As main functions, saliva participates in the mastication, deglutition, speech, lubrication, with functions in ionic balance regulation, and antibacterial, antifungal and antiviral activities [109].

There is a growing interest in the use of human whole saliva for diagnostic and disease monitoring as an alternative to blood samples [107]. Consequently, a large number of analytes in saliva were gradually unveiled and some are biomarkers of different diseases [110].

The utility and usability of saliva as diagnostic fluid has been studied by researchers and several Omics saliva studies have led to the production of massive amounts of data collected and annotated in databases on the identification and characterization of the different salivary components (DNA, RNA, proteins, metabolites and microorganisms) [111]. Saliva reflects the health or disease state [112-123] of an individual. The advantages of using saliva relatively to other fluids (blood, serum or plasma), which include the simple, non-invasive and safer sampling methods that require minimal equipment and easy and inexpensive storage possibilities.

Contrary to other sterile fluids, like the cerebrospinal fluid [124], blood, amniotic and pleural fluid, saliva is not sterile [107] and therefore it is subjected to microbial degradation which influences sample quality [125]. Nonetheless the effect of saliva collection, preparation and storage methods on the amount of microbial derived degradation hasn’t been extensively studied. Furthermore, some parameters such as diurnal, inter and intra individual variation on the different components haven´t been definitely established. In fact, even though for some proteins and steroid hormones there are data supporting diurnal variations [126-128] for other proteins, total volume and protein concentration [126, 129] the quantifications seem to be independent of the circadian cycle [127].

1.4 Biobanks

Biobanks comprise organized collections of biospecimens (tissue, blood, urine, plasma, saliva) annotated with personal and clinical information and are a fundamental resource for high quality academic research and translational medicine [130, 131]. The development and validation of analytical methods, diagnostic tests and biomarker discovery depends on good quality repositories [124]. Two main concerns of biobank managers are sample preservation and annotation quality and consistency, both of which are dependent on the standardization of the collection, processing and storage protocols [130].

There are different types of biobanks depending not only on the biospecimens they store, but also on the research purposes they serve [124]. Biobanks range from project driven biobanks to more general repositories which function as a reference collection to address different and not predetermined questions or specific research purposes [132].

In the 2000s about 43 biobanks were created around the world [124]. In Portugal the major Biobank is established in the Lisbon Academic Medical Centre [133]. This biobank has been previously described [134] and includes mainly blood derived specimens. Recently, as the result of a partnership with the Departamento de Ciências da Saúde of the Universidade Católica Portuguesa, it has started to store saliva samples.

1.5 Salivary Biomarkers

Efforts are made to development and validation of biomarkers to facilitate the identification of novel and effective treatment and strategies for several diseases. [135] Biomarkers are entities within the body capable of providing information regarding the current physiological state of an organism [136]. By definition, a biomarker is a biological characteristic that is objectively measured and evaluated as an indicator of normal biological or pathologic processes [137]. These

biomarkers exist in a variety of forms like antibodies, microbes, DNA, RNA, lipids, metabolites and proteins [138]. Potential biomarkers discovered in omic libraries (proteomic, transcriptomic, metabolomic, epigenomic) must be subjected to comprehensive evaluation and validation [139]. A biomarker should be reliable, reproducible, noninvasive, simple to perform and inexpensive. The onset, progression or regression of a disease can be associated with the alteration of the concentration, structure, function, or action of biomarkers [138]. Concluding, a biomarker is a valuable and attractive tool for detection, risk assessment, diagnostic, prognostic and monitoring of disease [140]. The analysis of protein levels as a source of differentially expressed proteins that represent biomarkers has become an established field of research [141]. Protein biomarkers can be studied in neuropsychiatric disorders [142].In those cases, proteomic research is focused on comparing protein expression levels between patients and healthy controls.

1.5 The Bioinformatic tool OralCard

Bioinformatics has became a central core that integrates the disparate bodies of data, scientific knowledge and computational infrastructure from genetics, structural biology and medical and animal models of disease [143]. OralCard is a bioinformatic tool dedicated to scientific research in oral health; this tool allows the analysis and integration of data from oral proteomes, contributing to the elucidation of oral biology and to the design of strategies for the identification of biomarkers for oral and systemic diseases [111]. OralCard collects information on proteins present in the oral cavity [111]. When searching for protein name or its UniProt code, OralCard retrieves information about the protein providing associations between proteins, diseases, pathways, gene ontologies and organisms. Integrating all this information, allows the user to perform queries in a fast and intuitive way [111]. In this dissertation, OralCard is used since it gathers data about the proteome of the oral cavity and because proteins are used as potential biomarkers. In the context of this study, OralCard will be updated with proteins that are associated with neuropsychiatric diseases will be carried out.

2. Objective

The objective of this dissertation is to establish the methodology for identification of inflammatory molecular markers in ADHD and dyslexia using unstimulated whole saliva as a diagnostic body fluid.

In order to fulfill the objectives, a series of tasks will be performed:

- ✓ Establish the methodology to create a standard operating procedure (SOP) for the collection, the processment and the quality control of saliva samples.
- ✓ Update OralCard database with proteins reported to be involved in neuropsychiatric diseases.
- ✓ Compare the overall profile of proteins present in the saliva of healthy donors with ADHD and dyslexia.
- ✓ Contribute to the establishment of a collection of saliva samples of children with ADHD and dyslexia within the Instituto de Medicina Molecular (IMM) collection biobank.
- ✓ Evaluate two chemokines CCL3 e CCL13 which are related with the inflammatory response.

3. Materials and Methods

In the flowchart below **(Figure 8)** the methodology used in this study is summarized.

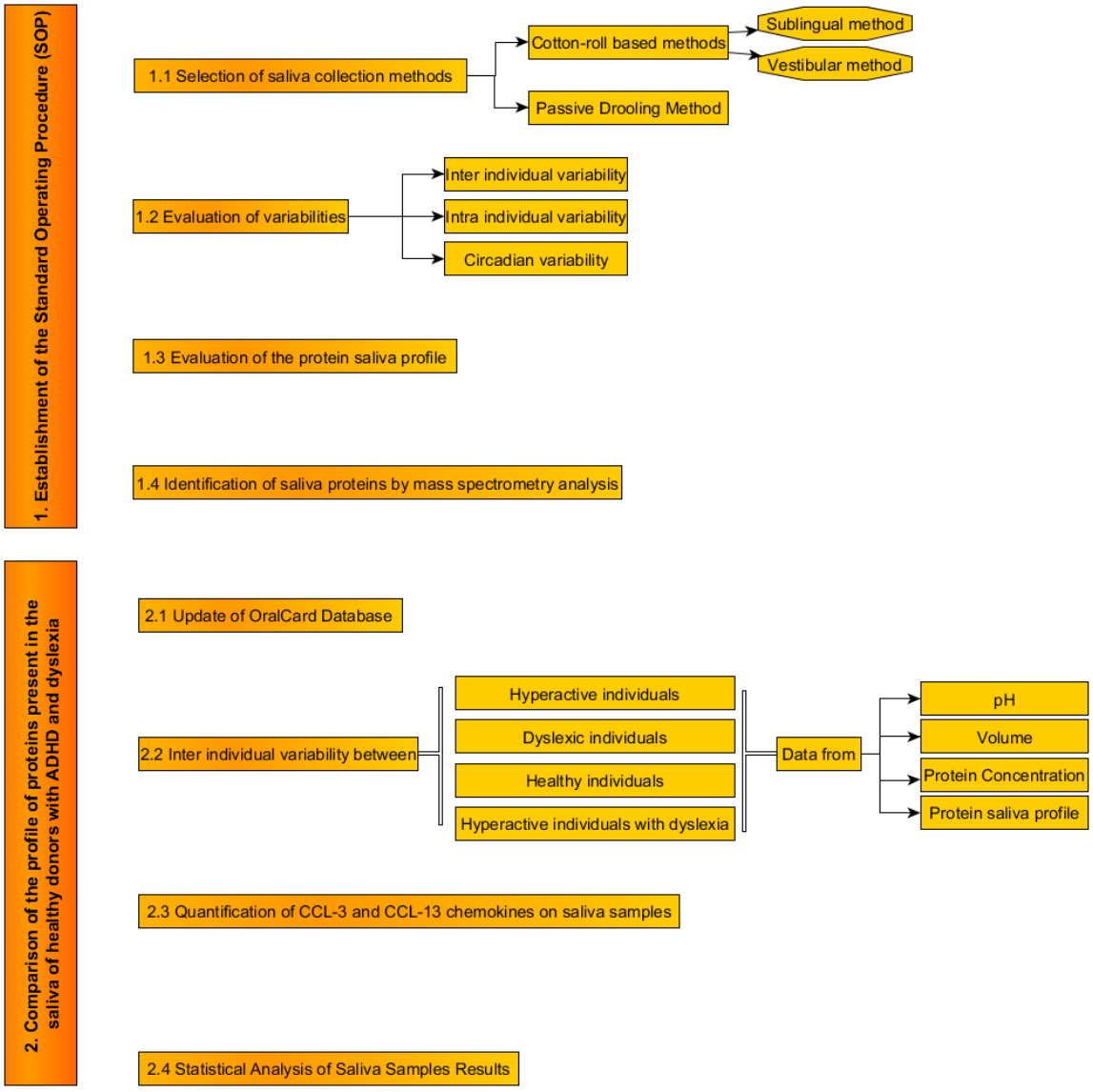


Figure 8 - Flowchart that shows the work developed. The first part concerns the establishment of the SOP and the second part the comparison of the different profiles of samples (healthy, hyperactivity, dyslexia and multi-compromised individuals). Image prepared with yEd Graph Editor version 3.14.2.

There are several methods to collect saliva samples [112, 144-146]. However, despite several published methods a comparison of several methods to establish

one protocol wasn´t available. For the establishment of the SOP, it is necessary to select a collection method and site as well as the characterization of the impact of the circadian variability on the volume, protein concentration, pH and protein profile of the collected saliva. The evaluation of inter and intra individual variability complements the evidence necessary to establish a SOP guaranteeing the preliminary quality control characterization of the saliva.

3.1 Participants and ethics statements

For the establishment of the SOP, saliva samples were collected at Universidade Católica Portuguesa on 22 healthy volunteers (9 males and 13 females) aged between 19 and 27 years old (mean=21 years; SD=2.34). This is a convenience sample representative of the university students.

For the identification of inflammatory biomarkers in ADHD, saliva samples were collected at Centro de Hiperatividade and Centro de Dislexia of Universidade Católica Portuguesa and in the Agrupamento de Escolas Viseu Sul on 27 volunteers aged between 7 and 16 years old (mean=12 years; SD=2.27). The study population was composed of 13 males and 14 females. Many hyperactive individuals may also have dyslexia. The creation of a group with dyslexia was extremely important to distinguish these two pathologies which are often associated. The 27 subjects were divided into four groups: 1-healthy subjects (7); 2- hyperactive individuals (10); 3- dyslexic individuals (5) and 4- individuals with hyperactive and dyslexia (5).

Donors or their legal representatives consented to the collection and storage of the samples and associated data by signing an informed consent **(Annex 1)** approved by the Ethics Commission of the Centro Hospitalar Lisboa Norte – Hospital de Santa Maria. The clinical database in which these data are deposited is authorized by the National Commission for Data Protection [133]. At the time of sample collection a questionnaire **(Annex 2)** was completed by all subjects/participants and archived with the respective serial number on a database created for this purpose as well as data that characterizes the samples. In the selection of participants, the exclusion criteria were presence of any systemic illness and

antibiotic medication during the previous 3 months. The control group was composed by healthy individuals with the same age of the groups with the pathologies.

3.2 Saliva sample collection methods

For the comparison of collection methods, unstimulated whole saliva of 8 healthy subjects was collected in the morning and in the afternoon in two different days with a total of 36 samples. Subjects were asked to refrain from eating, drinking or have oral hygiene procedures 1h prior to saliva collection. Immediately before saliva collection, the subjects were asked to rinse the mouth with clean water for 30 seconds. This cleaning step is crucial to remove desquamated epithelial cells, food and drink remnants. After the mouth rinse, subjects were asked to wait for a minute before collection was performed. Saliva was collected by three different methods: passive drooling, sublingual cotton-roll and vestibular cotton-roll [147].

3.2.1 Passive drooling method

In this method, a 50mL sterile tube was used to collect passive drooled saliva for 3 minutes. The 50mL tube was maintained on ice during collection to ensure the integrity of the sample.

3.2.2 Cotton-roll based methods

In these methods sublingual or vestibular saliva was collected with two cotton rolls which were placed under the tongue or the vestibular area respectively, for 2 minutes. The cotton rolls were collected and placed inside a 15mL sterile plastic tube with a sterile P100 pipette tip in the bottom **(Figure 9)** to facilitate saliva collection by a centrifugation step at 10000 x g for 10min at 4ºC.

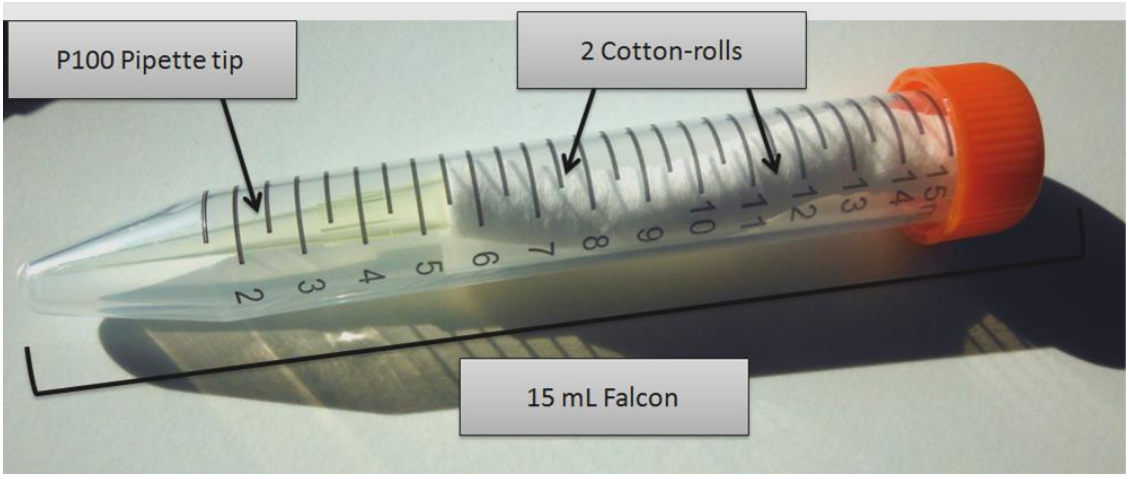


Figure 9 – Test tube used for the collection of saliva samples.

Each sample was characterized as to total volume collected and protein concentration. After re-suspension by vortex the total volume is divided in 50µl aliquots stored at – 80ºC.

3.3 Selection of saliva collection methods

The characterization of the saliva samples obtained with the three collection methods was carried out on 8 samples from healthy subjects based on the total volume of saliva and the protein concentration.

Saliva volume was measured. Protein concentration was determined using 5µL of each sample with the protein ultraviolet (UV) program of a NanoVue Spectrophotometer (Life Science, GE Healthcare, UK). NanoVue can determine protein concentration at 280 nm and use the equation: Protein (mg/mL) = 1.55 ×Abs280nm – 0.76.

3.4 The inter and intra-individual variability and the circadian effect

Upon the establishment of the collection procedure (sublingual method), inter-individual variation and the circadian effect regarding saliva volume and protein concentration were assessed. The number of subjects where increased to 22 (the

same 8 subjects more 14 other subjects) to gain statistical power. Protein concentration and volume were evaluated individually comparing morning and afternoon samples with 22 healthy subjects.

The evaluation of the intra and inter individual variability was performed on the same 8 healthy subjects increasing the morning collection in 11 different times (during 5months). Total volume and protein concentration were determined.

A Cluster to compare variability between individuals was performed. To construct the dendrogram we used the program PermutMatrix 1.9.3, the algorithm Ward's and the data about the protein concentration obtained by capillary electrophoresis and the molecular weight (MW) [148].

3.5 Evaluation of the protein saliva profile

Capillary electrophoresis using a Experion Automated Electrophoresis System (BioRad) with standard protein chips (Experion™ Pro260 Analysis Kit, #7007102, BioRad) were used to evaluate the electrophoretic protein profile of each sample and for protein band quantification. The chip runs were performed according to BioRad technical specifications. Briefly, to all saliva samples sample buffer (#7007102, BioRad) with β-mercaptoethanol was added. The saliva samples and the ladder (#7007102, BioRad) were subjected to the same denaturing conditions (95º-100ºC for 3-5min). The ladder ranges from 10 to 150kDa. The dilutions of saliva samples were done with ultrapure water. The migration times and the concentration of each protein in the sample wells were normalized to the ladder using internal markers present in each sample and in the ladder well.

3.6 Identification of saliva proteins by mass spectrometry analysis

For protein processing, samples were denatured using Laemmli buffer (BioRad) containing sodium dodecyl sulfate with dithiothreitol, alkylated with acrylamide and resolved by gel-electrophoresis (short-GeLC [149]).

Entire gel lane was sliced, digested with trypsin and peptides were extracted. This complex mixture of peptides was then analyzed by LC-MS/MS micro-reversed-phase at low pH coupled to a high resolution mass spectrometer (Triple TOF^{TM} 5600 ABSciex®). Peptide fragmentation spectra were generated for protein identification using ProteinPilot software (ABSciex®) against Uniprot.

3.7 Update of OralCard database

A review of the literature related to proteins involved in neuropsiquiatric diseases was made. A literature search was performed using the search engine aid NCBI - PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and with the read cube. For the research were used keywords such as ADHD, autism spectrum disorders, proteomics, neurodevelopmental disorderd and schizophrenia. To analyze and visualize graph-oriented genomic complex networks in which these proteins are involved, the Cytoscape application version 3.2.1 with ClueGO+CluePedia was used.

3.8 Inter individual variability of saliva samples from hyperactive, dyslexic and healthy individuals

The sublingual saliva samples were processed according to the SOP established and for all samples total volume, protein concentration and pH were determined.

A pre-evaluation of sample quality was performed by capillary electrophoresis using the Experion software version 3.20 (BioRad) with standard protein chips to evaluate the integrity of total protein profile.

To verify if there is a protein profile characteristic of each pathology, in multicompromised individuals with ADHD and dyslexia, a dendrogram of each protein profile was built. To construct the dendrogram we used the program PermutMatrix 1.9.3, the algorithm Ward's and the data about the protein

concentration obtained by capillary electrophoresis and the molecular weight (MW) [148].

3.9 Quantification of CCL3 and CCL13 chemokines on saliva samples

The multiplex, a reliable technique established on urine [150], saliva [151, 152], plasma [153-158], cerebrospinal fluid [158] and blood cultures [159] was used. Samples were assayed using multiplex technology, enabling the evaluation and quantification of the proteins according to manufacturer's guidelines, using a 96 well plate with fluorescent coded magnetic microspheres coated with analyte specific capture antibodies. After microspheres have captured the analytes, a biotinylated detection antibody binds to that complex. Streptavidin-phycoerythrin then attaches as a reporter molecule. Inside the instrument, magnetic beads are held in a monolayer by a magnet, where two lights emitting diode are used to excite the internal microsphere dye and the dye of the reporter molecule, respectively. A charge-coupled device camera captures these images, which are then analyzed by BioPlex Data Pro™ software. The human chemokine assay to evaluate and quantify CCL3 (Bio-Plex Pro^{TM} Human Chemokine MIP-1α/CCL3 Set#171BK44MR2) and CCL13 (Bio-Plex Pro^{TM} Human Chemokine MCP-4/CCL13 Set#171BK39MR2) proteins was used. 15 µl of each sample was used (diluted 1:4 v:v).

3.10 Statistical analysis

The statistical analysis was done with GraphPad Prism 6 (GraphPad Software, USA) and $p<0,05$ was used as cut-off for significance. The comparison of collection methods was performed on morning and afternoon collections of each method with two replicates with a total of 36 saliva samples. In terms of total volume, non-parametric Freadman Test was done and Dunn's test for multiple comparisons. For protein concentration, since the data have a normal distribution, parametric one way ANOVA was done and for multiple comparisons the Tukey test was used. To evaluate if the total volume and total protein are influenced by the circadian effect, a paired t-test was done with morning and afternoon samples

of 22 individuals. To compare inter-individual and intra-individual variability a two way ANOVA was done. After that, the coefficient of variance was measured to compare the variance of the proteins that vary.

With the samples individuals with ADHD, to compare the four groups of hyperactivity, dyslexia, hyperactivity with dyslexia and healthy in terms of volume, protein concentration and pH, Kruskal-Wallis test of one-Way ANOVA was done. Then, with the output data of capillary electrophoresis, a Kruskal-Wallis test of one-Way ANOVA was done to do a band-to-band comparison based on the different MW (from 9kDa to 240kDa) between the different groups. Then we used the Dunn's multiple comparison test to compare the protein concentration mean of each group with the control group that is the healthy group.

The statistical analysis of data obtained by multiplex technology from CCL3 and CCL13 proteins was done by one-way ANOVA. A P value was calculated based on Kruskal-Wallis test. The multiple comparisons were done with the Dunn's multiple comparison test.

3.11 Biobank associated data from individuals and samples

Associated to biobank the IMM saliva collection a database with clinical information and the quality of life was developed. The serial number of each sample was deposited on an online application named Quartzy (https://quartzy.com/) that is an online system for laboratory management **(Figure 10)**. At Quartzy, each sample is associated to its collection day and time, method of collection, pH, volume, protein concentration, the quantity of sample in µl, the gender, the birth date and its location on the biobank.

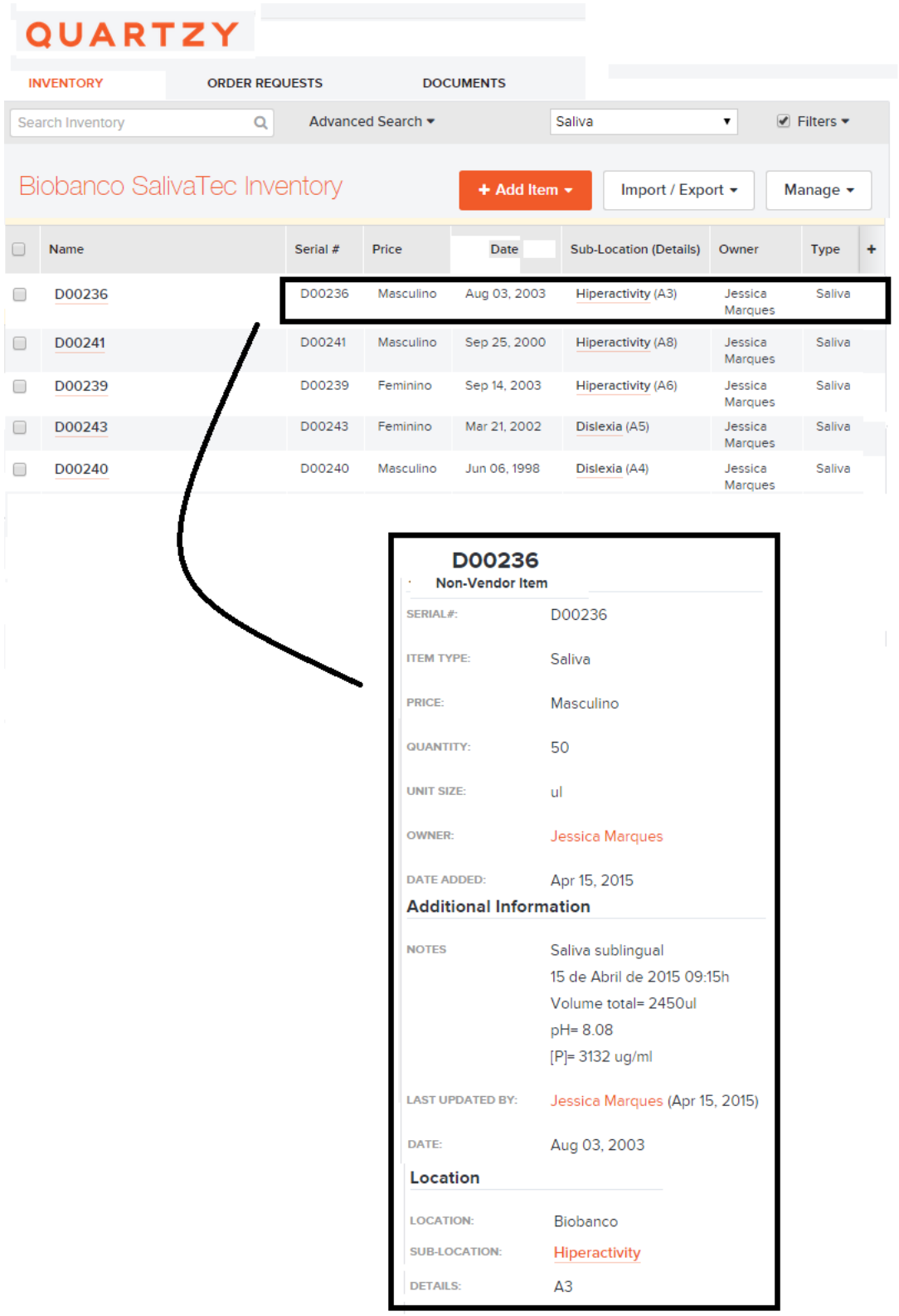


Figure 10 – Example of a Quartzy entry.

In addition the general information about the donors (birth date, gender, biometric data, ethnic, residence area, marital status, education level and profession, personal habits of smoking, drinking, exercise and eating habits; disease and allergies development, medication, genetic predisposition, lifestyle, scales such happiness and satisfaction of life) were assessed through a questionnaire **(Annex 2)**. This information was also deposited at Qualtrics (www.qualtrics.com/), a software-as-a-service company that offers an online platform for generating online surveys **(Figure 11)**.

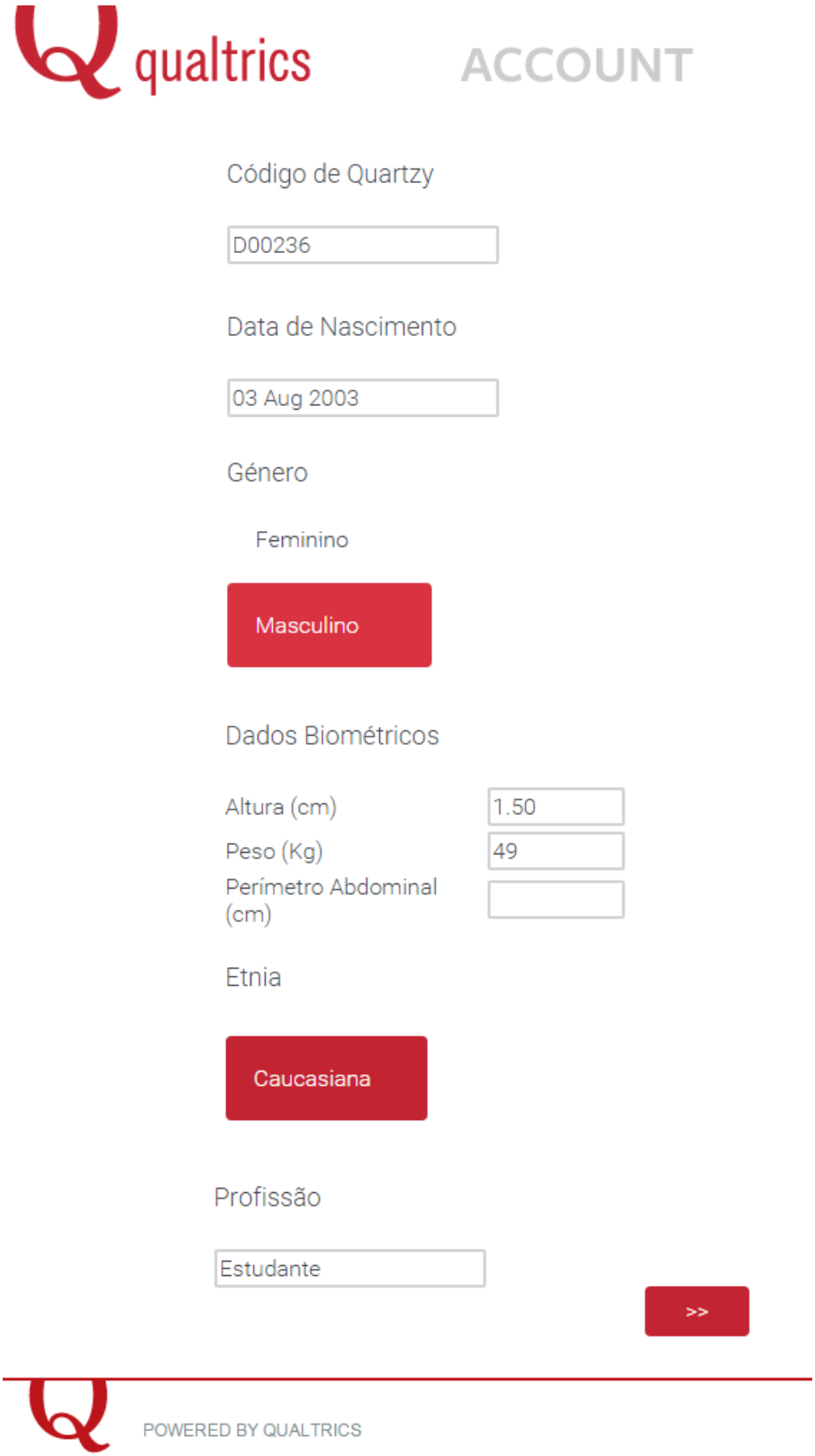


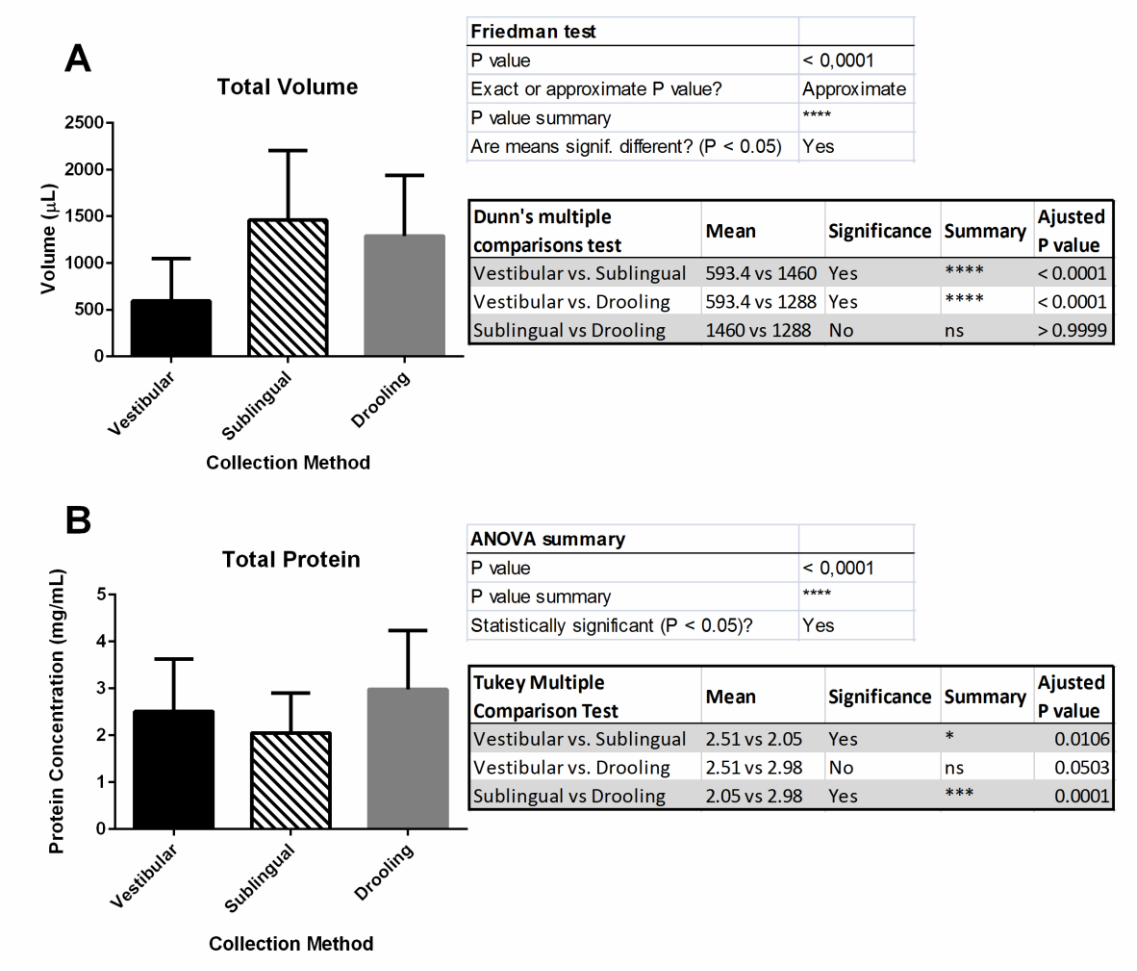
Figure 11- Example of a Qualtrics entry

4. Results and Discussion

4.1 Establishment of the standard operating procedure for saliva samples

4.1.1 Selection of a saliva collection method

Comparing the vestibular, sublingual and drooling saliva collection methods significant differences between the sublingual and the vestibular methods were found. Total volume **(Figure 12A**; p<0.0001**)** was significantly different between the 3 methods, while protein concentration was not **(Figure 12B)**. In terms of volume, there is also a significant difference between vestibular and drooling with a p<0.0001 **(Figure 12A)**. In terms of protein concentration, there is a significant difference between sublingual and drooling with a p=0.0001 **(Figure 12B)**.



Friedman test	
P value	< 0,0001
Exact or approximate P value?	Approximate
P value summary	****
Are means signif. different? (P < 0.05)	Yes

Dunn's multiple comparisons test	Mean	Significance	Summary	Ajusted P value
Vestibular vs. Sublingual	593.4 vs 1460	Yes	****	< 0.0001
Vestibular vs. Drooling	593.4 vs 1288	Yes	****	< 0.0001
Sublingual vs Drooling	1460 vs 1288	No	ns	> 0.9999

ANOVA summary	
P value	< 0,0001
P value summary	****
Statistically significant (P < 0.05)?	Yes

Tukey Multiple Comparison Test	Mean	Significance	Summary	Ajusted P value
Vestibular vs. Sublingual	2.51 vs 2.05	Yes	*	0.0106
Vestibular vs. Drooling	2.51 vs 2.98	No	ns	0.0503
Sublingual vs Drooling	2.05 vs 2.98	Yes	***	0.0001

Figure 12– Comparison between the three collection methods tested: volume of saliva collected (A) and sample protein concentration (B). Volume was analyzed by non-parametric Freadman test (Dunn's test for multiple comparisons) and protein concentration was analyzed by parametric one-way ANOVA (Tukey test for multiple comparisons). Statistical analysis was performed on data from 36 saliva collections for each method, with morning and afternoon collections.

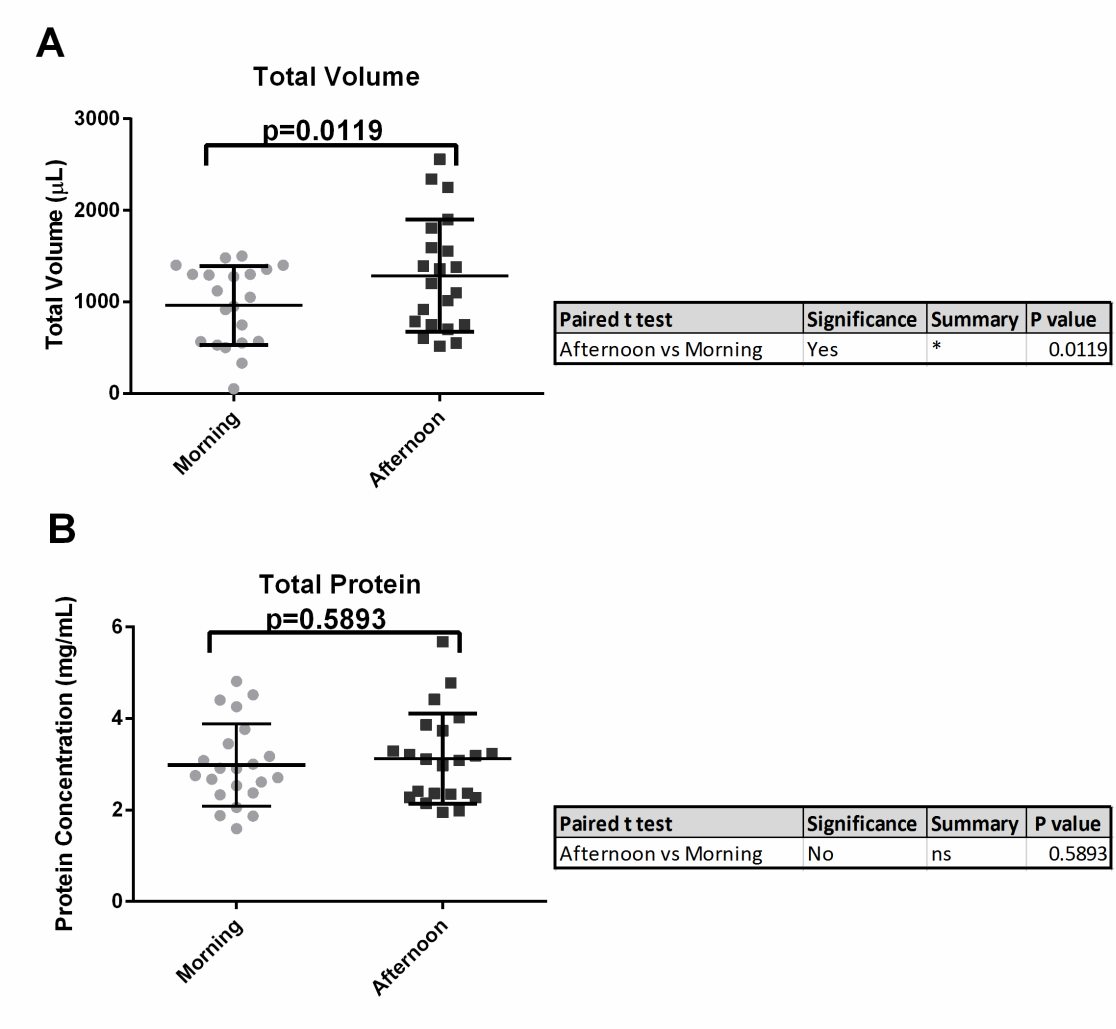
Considering these results, the sublingual cotton-roll method for saliva collection was selected for the subsequent saliva collection since it ensures a suitable sample volume and protein concentration in only in 2 minutes. This method is easy to carry out and friendly to the patient; furthermore, the fact that the cotton-roll acts like a filter helps to eliminate cell debris and protein aggregates, allowing a good quality sample. It is an easy method to perform in any laboratory since it requires minimal non expensive materials and equipment which is an advantage in large population studies. Nowadays, there are several oral fluid collector and commercial devices [160] available on the market, however these are expensive.

The sample volume and protein concentration, are important factors to have into account when saliva samples are collected. A good method should ensure a good volume (>1mL) and a good protein concentration (>1000μg/mL) of samples. The effect of the collection method on protein concentration should be evaluated and considered.

Results from the paired t-test comparing the circadian effect on morning and afternoon collections are presented in Figure 13 in terms of the effect on the volume **(Figure 13A)** and protein concentration **(Figure 13B)**.

Our results didn't show any difference between morning and afternoon collections in terms of protein concentration. However there are differences in terms of collected volume, wherein the afternoon sample volume mean is larger than the morning sample volume. There are many studies of circadian variations in the salivary flow rate and concentrations of particular ions of human saliva. For some proteins like like histatin1,3 and 5, cortisol and statherin and steroid hormones there are data supporting diurnal variations [126-128] while for other proteins, total volume and protein concentration in terms of the quantifications seems to be independent of the circadian cycle [127]. The collection must be done in the

morning since for certain proteins like alpha-amylase, cortisol, histatins and statherins there is a circadian rhythm associated [126-128].



Paired t test	Significance	Summary	P value
Afternoon vs Morning	Yes	*	0.0119

Paired t test	Significance	Summary	P value
Afternoon vs Morning	No	ns	0.5893

Figure 13- Circadian variability regarding sample volume (A) and protein concentration (B) for morning and afternoon collections. Statistical analysis was performed by Paired t test. Statistical analysis performed on data from 22 morning saliva collections.

4.1.2 Inter and intra individual saliva characteristics variability

Comparing inter and intra individual variation, the results for volume show that there is statistical significance between individuals ($p<0.0001$) and in intra

individual variability (p<0.0001) **(Figure 14A)**. For protein concentration there is statistical significance between individuals (p <0.0001) and there is also statistical significance within the same individual (p<0.0001) **(Figure 14B)**. In terms of total variation, the inter-individual variability overlaps the intra-individual variability in both total protein and volume.

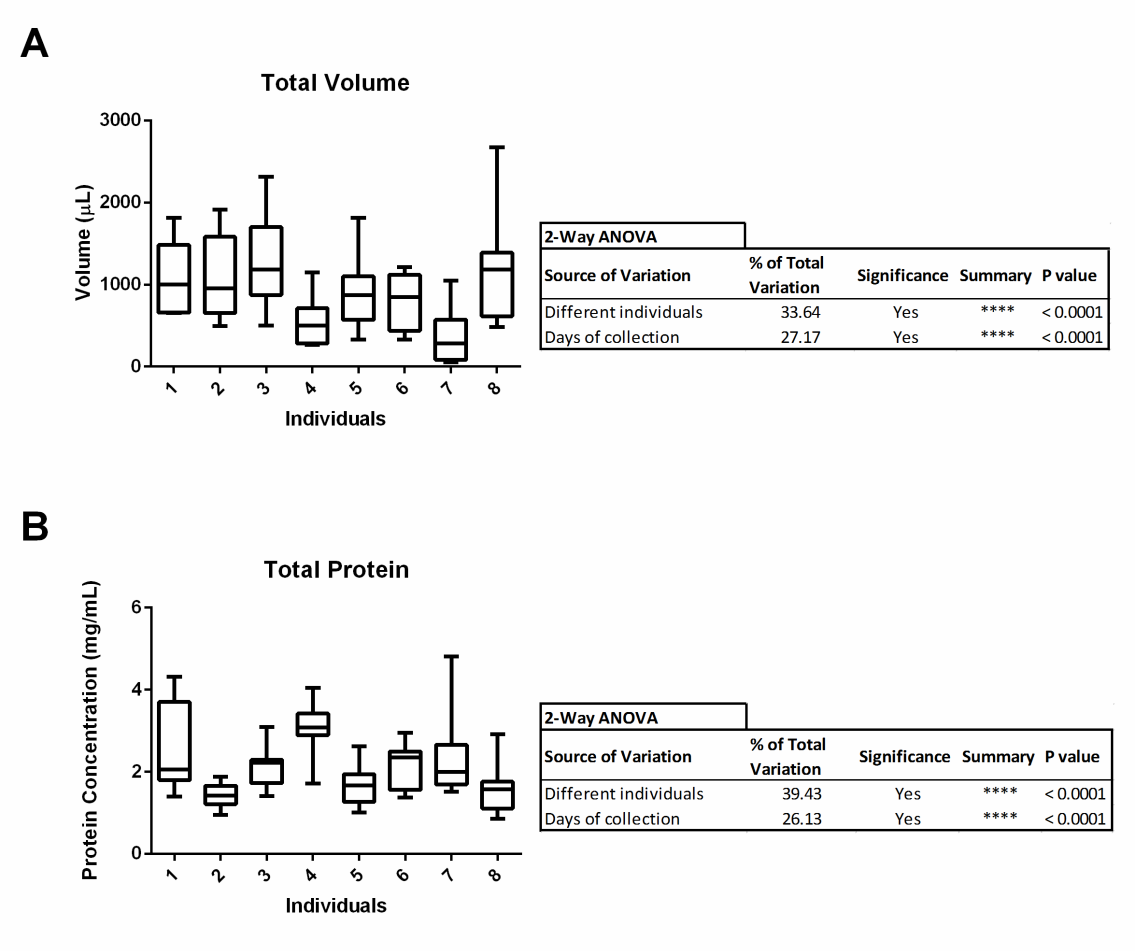


Figure 14- Box-plot of inter individual and intra individual variability on sample colume (A) and protein concentration (B). The results correspond to saliva samples from 8 donors from which samples were collected at 11 different times (during 5 months). Statistical analysis was performed by a two-Way ANOVA.

The electrophoretic protein profile of the same 8 healthy individuals analysed above is shown in **Figure 15** to ascertain if there is a distinct protein profile in healthy individuals.

After the protein profile analysis, it was verified that some bands are common for all subjects (bands with 64 and 70 kDa, Figure 15 grey arrows) and others vary widely (37 and 30 kDa, Figure 15 black arrows).

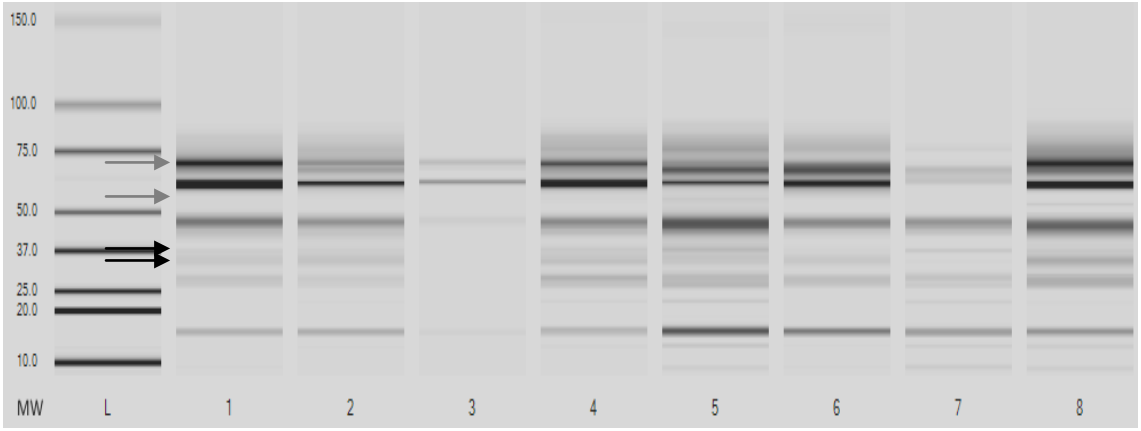


Figure 15- Inter-individual variability of protein electrophoretic profile. The protein profile from 8 different individuals (the same of Figure 7) analyzed by capillary electrophoresis using the Experion BioRad System. (L) Ladder with a range from 10 to 150 kDa.

From the previous results we concluded that there is an inter individual variability. Therefore, we analyze the variance of the protein profile of the 22 subjects considering samples collected on morning **(Figure 16)**. The concentration of each band is shown. The variability of the different bands is shown for the different MW. The proteins that appear in all individuals (white circles) are in the 13, 16, 28, 47 and 62 kDa range.

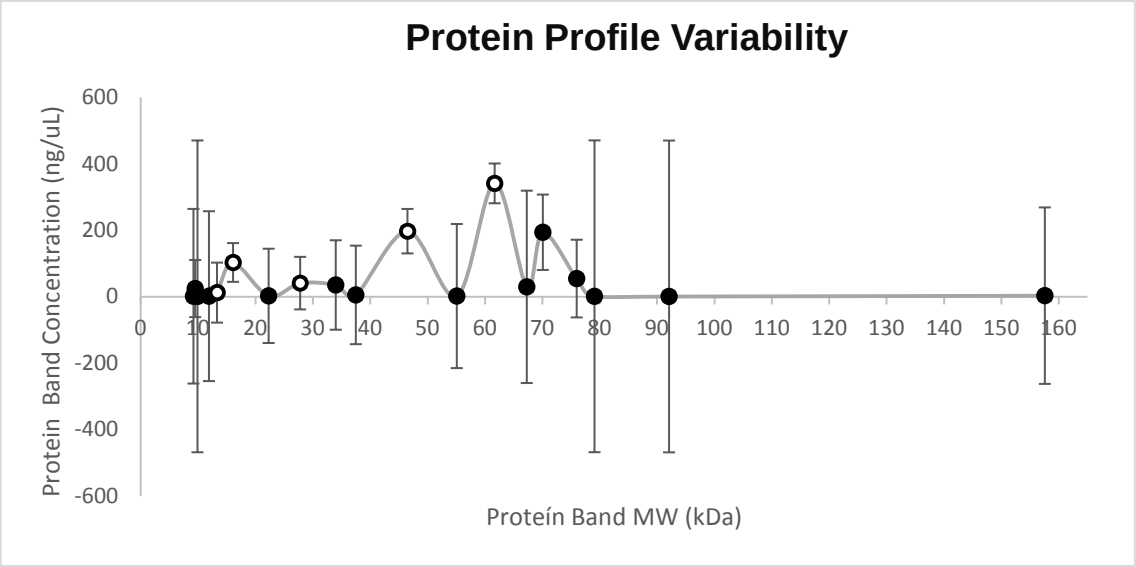


Figure 16- Distribution of protein concentration for each MW corresponding to the different bands obtained in the electrophoretic profile. The bars show the % of coefficient of variance (CV) for each group of proteins. The white circles identify the proteins that appear in all individuals studied.

Samples of 22 healthy individuals were used to identify the more distinct groups of proteins. The identification of inter individual protein profile variability is important to determine which proteins remain relatively unchanged in different individuals and which proteins vary widely. Proteins that are common to all samples regardless of the individual are good targets to look for variations between healthy and sick individuals.

The clusters obtained according to the protein profile are present in **Figure 17**. The columns represent the different individuals (1-22) and the rows different MW bands. This representation shows that there are two main groups of profiles and these are divided into others groups of closer profiles.

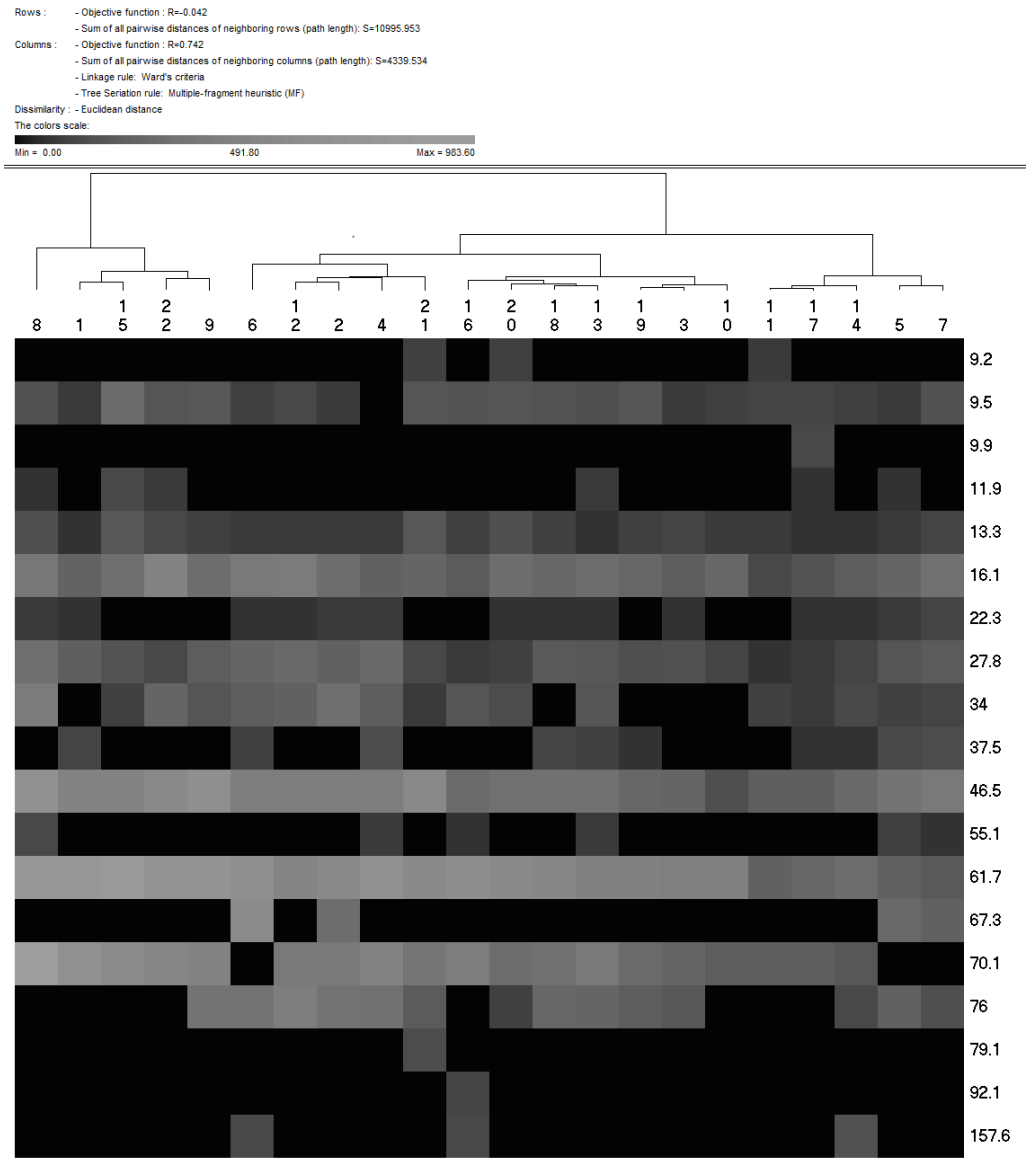


Figure 17- Heatmap visualizing the level of 19 bands differentially represented between subjects. Each column represents the data of protein concentration for all subjects. Rows represent the different MW. The color code is graduated from black (under-representation compared to the group mean) to grey (over-representation compared to the group mean). Hierarchical clustering analysis was used to organize the map. The Cluster is done with the PermutMatrix program using the Ward's minimum variance method (n=22).

4.1.3 Identification of proteins present in saliva

Saliva proteins were identified by mass spectrometry. A pool of 1012 peptides that correspond to 60 human proteins was identified and are presented in **table 1**. The

proteins are organized by their MW and UniProt code. The table also shows the proteins that are previously identified by other authors and are catalogued in OralCard database [111].

After that, with the proteins identified by MS in saliva samples a Cytoscape network was made with the objective of knowing the biological processes in which the proteins are involved. The pie chart of biological processes is presented in **Figure 18**.

The majority of proteins are involved in defense responses to fungi (left side of pie chart in purple), others in nicotinamide adenine dinucleotide metabolic process (right side of pie in fuschia) and in positive regulation of respiratory burst (pie chart in red). There are also proteins involved in retina homeostasis, detection of chemical stimulus, tissue regeneration, platelet degranulation and inflammatory response to antigenic stimulus.

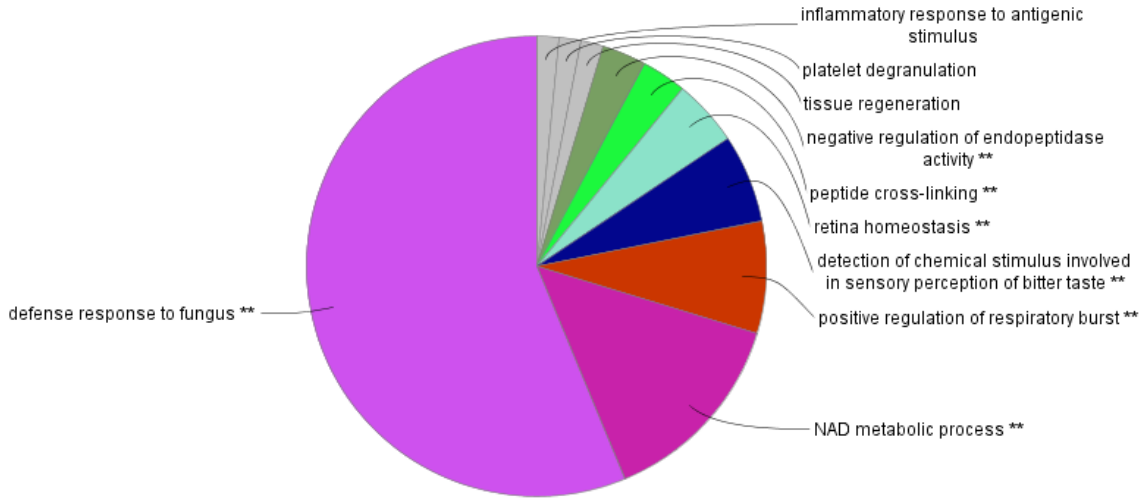


Figure 18- Pie chart of the human GO: Biological Processes represented on ClueGO+CluePedia of the proteins identified by mass spectrometry. The ** represents that there are a statistical significance of enrichment; the proteins presented in these processes are significantly increased in relation to the others. Image generated by Cytoscape (ClueGO+CluePedia) on June 22, 2015.

In a health situation these proteins are involved in these 10 processes. What is expected is that some proteins may be increased/decreased or absent/present in disease situations.

Table 1- Human salivary proteins identified by Mass Spectrometry analysis. The table shows the protein according to UniProt Code, MW, proteins deposited in the OralCard [111] and identified in whole saliva.

Mol. Wt. (kDa)	*UniProt Code*	*Protein Name*	OralCard	*Identified in whole saliva*
189	P01024	Complement C3 - fragment	x	x
161	A8K2U0	Alpha-2-macroglobulin-like protein 1	x	x
118	P22314	Ubiquitin-like modifier-activating enzyme 1	x	x
103	P55786	Puromycin-sensitive aminopeptidase (PSA)	x	x
86	P06396	Gelsolin (AGEL)	x	x
83	P01833	Polymeric immunoglobulin receptor (PIgR)	x	x
80	P22079	Lactoperoxidase (LPO)	x	x
78	P02788	Lactotransferrin (Lactoferrin)	x	x
77	**Q06AH7**	**Transferrin**		
77	Q08188	Protein-glutamine gamma-glutamyltransferase E	x	x
69	P02768	Serum albumin	x	x
69	P15311	Ezrin (Cytovillin)	x	x
58	P14618	Pyruvate kinase PKM	x	x
58	P04745	Alpha-amylase 1	x	x
57	P07237	Protein disulfide-isomerase (PDI)	x	x
54	Q9UBG3	Cornulin	x	x
53	P52209	6-phosphogluconate dehydrogenase	x	x
50	P80303	Nucleobindin-2	x	x
49	P01871	Ig mu chain C region	x	x
49	Q8N4F0	BPI fold-containing family B member 2	x	x
47	P06733	Enolase 1	x	x

Mol. Wt. (kDa)	*UniProt Code*	*Protein Name*	OralCard	*Identified in whole saliva*
47	P01009	Alpha-1-antitrypsin	x	x
44	Q9UIV8	Serpin B13	x	x
43	P30740	Leukocyte elastase inhibitor (LEI)	x	x
39	P04083	Annexin A1	x	x
38	P01876	Ig alpha-1 chain C region	x	x
38	Q6P5S2	Protein LEG1 homolog	x	x
37	P00338	L-lactate dehydrogenase A chain (LDH-A)	x	x
37	P01877	Ig alpha-2 chain C region	x	x
36	P01857	Ig gamma-1 chain C region	x	x
36	P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	x	x
35	P23280	Carbonic anhydrase 6	x	x
34	P25311	Zinc-alpha-2-glycoprotein (Zn-alpha-2-GP)	x	x
32	Q96DR5	Parotid secretory protein -fragment (PSP)	x	x
29	P18669	Phosphoglycerate mutase	x	x
29	P06870	Kallikrein-1	x	x
28	P31947	14-3-3 protein sigma	x	x
27	Q96DR5	BPI fold-containing family A member 2	x	x
23	P09211	Glutathione S-transferase P	x	x
23	P04792	Heat shock protein beta-1 (HspB1)	x	x
23	Q96DA0	Zymogen granule protein 16 homolog B	x	x
20	P18510	Interleukin-1 receptor antagonist protein (IL-1RN)	x	x
19	P31025	Lipocalin 1	x	x
18	P01591	Immunoglobulin J chain	x	x

Mol. Wt. (kDa)	*UniProt Code*	*Protein Name*	OralCard	*Identified in whole saliva*
18	Q9UHA7	Interleukin-36 alpha	x	x
18	Q9UBC9	Small proline-rich protein 3	x	x
17	P27482	Calmodulin-like protein 3	x	x
17	P12273	Prolactin-inducible protein (PIP)	x	x
17	P02810	Salivary acidic proline-rich phosphoprotein 1/2	x	x
16	P28325	Cystatin D	x	x
16	P09228	Cystatin-SA (Cystatin-2)	x	x
16	P01037	Cystatin-SN (Cystain-SA-I)	x	x
16	P01036	Cystatin-S (Cystatin-4)	x	x
16	P01034	Cystatin-C (Cystatin-3)	x	x
15	P07737	Profilin-1	x	x
15	Q01469	Fatty acid-binding protein	x	x
13	P06702	Protein S100-A9 (Calgranulin-B)	x	x
11	P01040	Cystatin-A (Cystatin-AS)	x	x
11	P05109	Protein S100-A8 (Calgranulin-A)	x	x
10	P07108	Acyl-CoA-binding protein (ACBP)	x	x
8	P02814	Submaxillary gland androgen-regulated protein 3B (Proline-rich peptide P-B)	x	x
7	P15515	Histatin-1 (Histidine-rich protein 1)	x	x

4.2. Update of OralCard database

A review of the literature of proteins related to neuropsiquiatric diseases was made. After this revision, a list with the names of the 56 proteins, UniprotKBAC code (obtained by http://www.uniprot.org), pathologies in which that protein arises increased or decreased relative to normal as well as information about the study was obtained **(Annex 4)**. Using the Uniprot codes of these proteins a network of the biological processes in which these proteins are involved is shown in **Figure 19**.

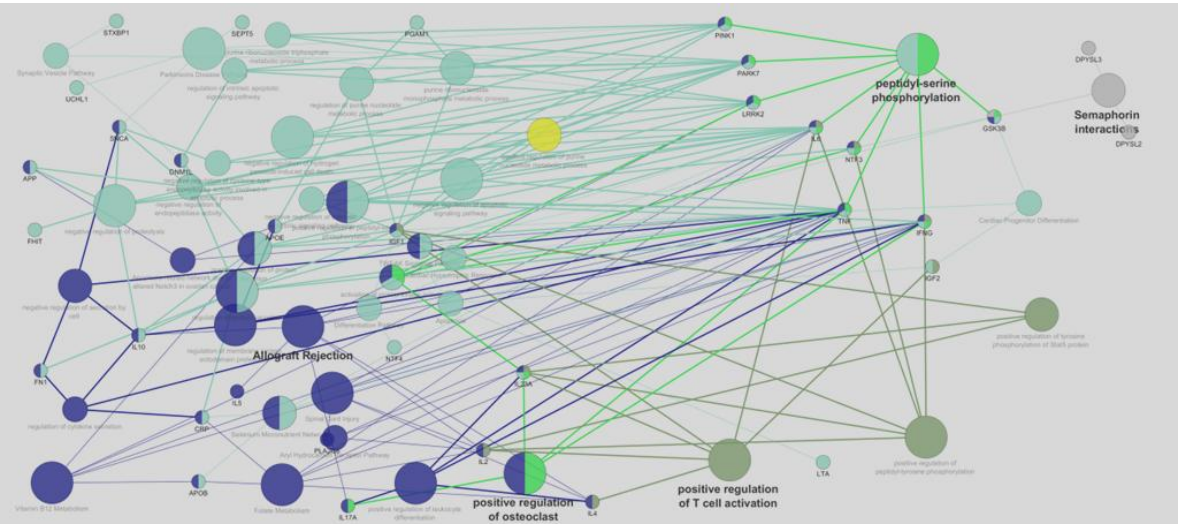


Figure 19 - Network of human GO: Biological Processes. At bold are the most prominent processes. The small circles represents the proteins and the bigger the processes in which the proteins are involved. The lines represent the interactions. Image generated by Cytoscape (ClueGO+CluePedia) on June 22, 2015.

The network of biological processes highlights a great commitment of the immune response in these pathologies, underlining the positive regulation of T cell activation and the positive regulation of osteoclasts. There are proteins involved in Parkinson's disease (PD), in neurotransmission (peptidyl-serine phosphorylation) and in allograft rejection. There are also proteins involved in semaphorin interaction. Semaphorins are a family of glicoproteins that mediate many cell processes critical to the immune system including cell-cell contact, migration and cytokine secretion [161]. Altered expression and function of semaphorin family members are associated with neurologic disorders and regenerative failure

following central nervous system injury [162]. Since that network is very complex, a detailed pie chart of the biological processes is made with the name of the proteins involved in a given process **(Figure 20)**.

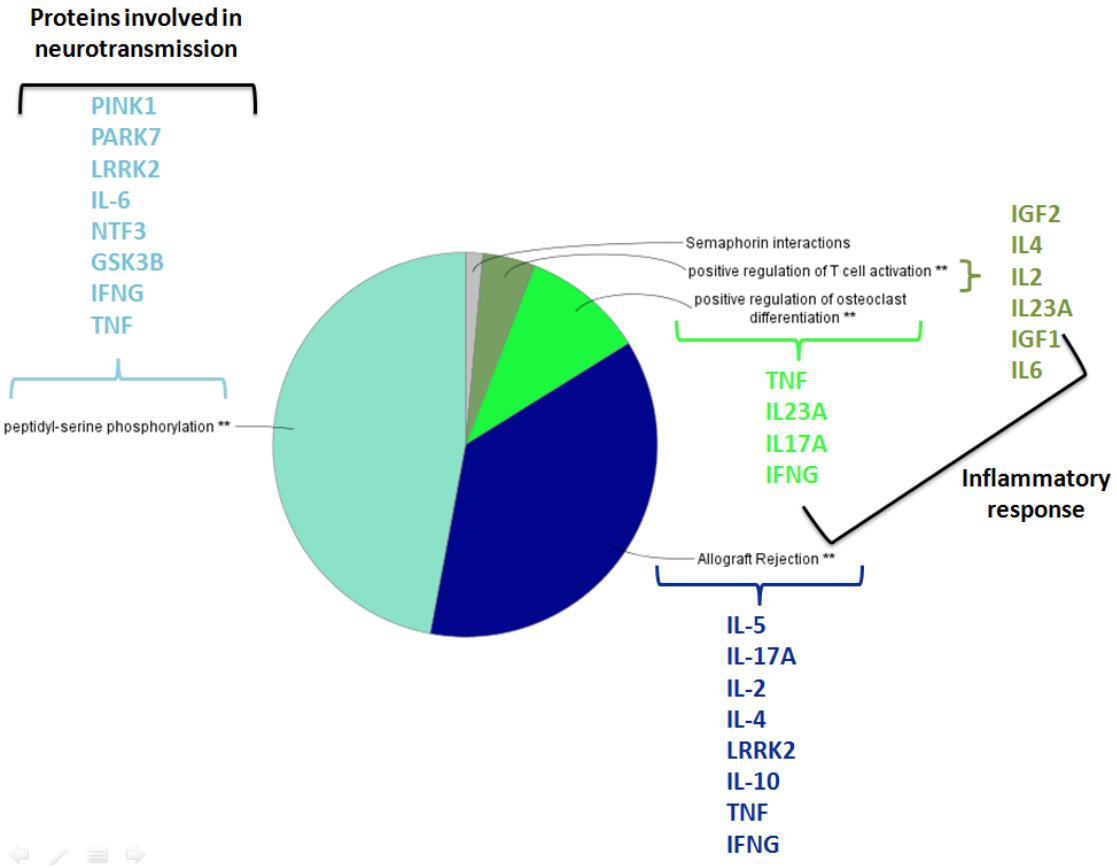


Figure 20 – Pie chart of the human GO: Biological Processes. At dark blue we have the allograft rejection, at light green the positive regulation of osteoclast differentiation, at dark green the positive regulation of T cell activation, at grey the semaphoring interactions and at light blue the peptidyl-serine phosphorylation. The ** represents that there are a statistical significance of enrichment; the proteins presented in these processes are significantly increased in relation to the others. Image generated by Cytoscape (ClueGO+CluePedia) on June 22, 2015.

With this result we have more detailed information on the proteins and biological processes. For example, some proteins like TNF and interferon G appear in three processes, in the allograft rejection, peptidyl-serine phosphorylation and positive regulation of osteoclast differentiation.

After that, a network of Wikipathways represented on ClueGO+CluePedia was made **(Figure 21)**. This network shows the processes and pathways in which the proteins are involved, namely the synaptic vesicle and PD and differentiation pathway as well as the cardiac hypertrophic response and allograft rejection.

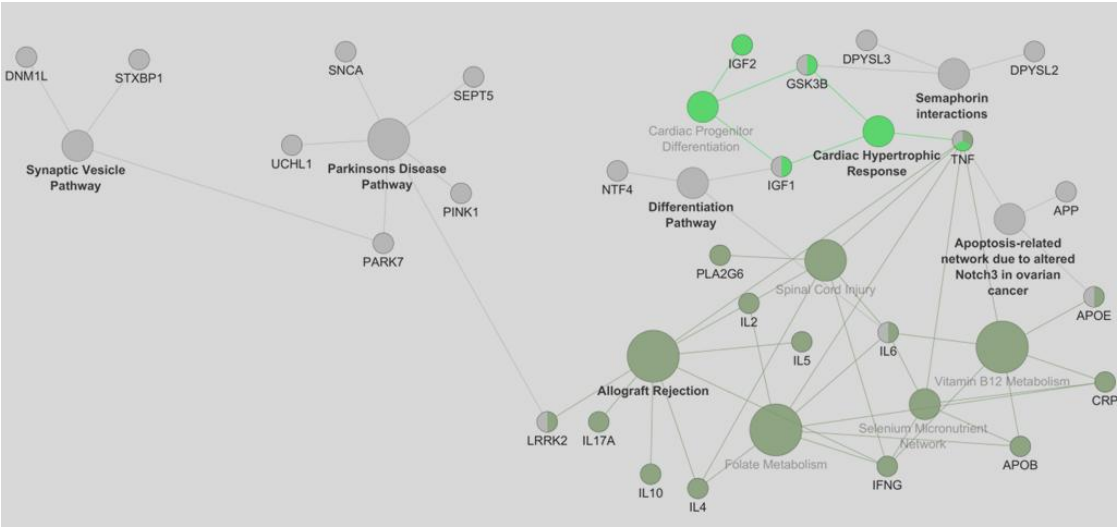


Figure 21- Wikipathways network. At bold are the most prominent processes. The small circles represents the proteins and the larger the processes in which the proteins are involved. The lines represent the interactions. Image generated by Cytoscape (ClueGO+CluePedia) on June 22, 2015.

In ADHD the lack of nutrients is a problem and a proper nutrition is essential for ADHD treatment. In this network the vitamin B12 metabolism, the selenium micronutrient network and the folate metabolism are evidenced. The low levels of folate during pregnancy are related with hyperactivity in children. Deficiencies in folate and vitamin B12 have been associated with neurodegenerative diseases [163], like vascular dementia, AD and PD [164]. Selenium has also been identified as playing a role in several neurodegenerative disorders, including AD and PD [165].

A network of Kyoto Encyclopedia of Genes and Genomes on ClueGO+CluePedia was also made **(Figure 22)**.

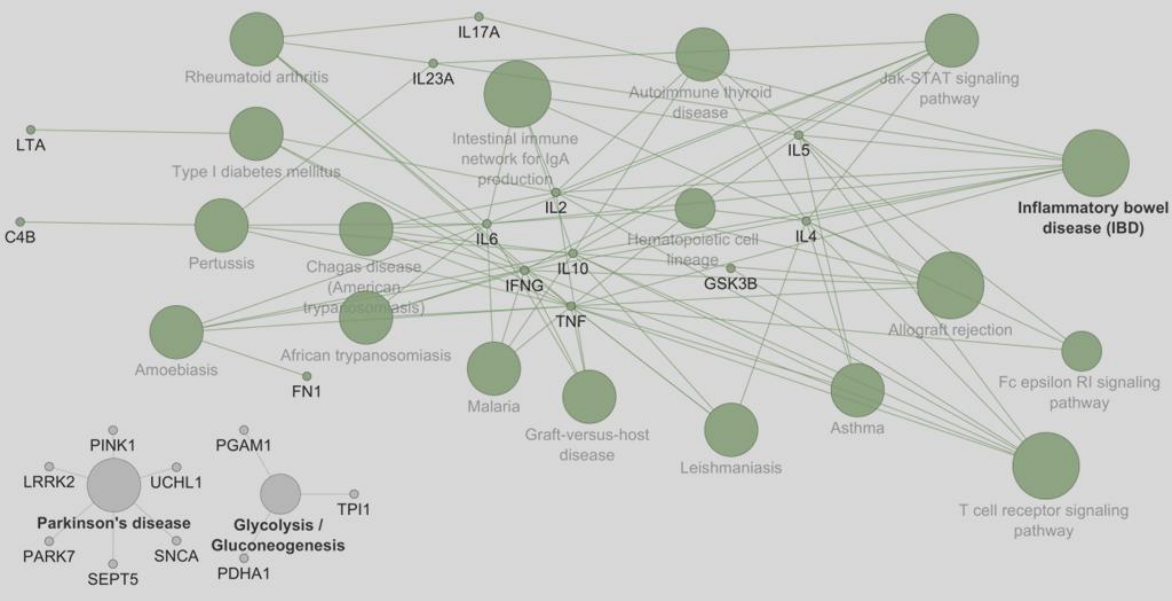


Figure 22- Network of Kyoto Encyclopedia of Genes and Genomes represented on CluePedia. The small circle represents the proteins and the larger circles the diseases and processes in which the proteins are involved. The lines represent the interactions. Image generated by Cytoscape (ClueGO+CluePedia) on June 22, 2015.

By the analysis of this network, the majority of the proteins are involved in a variety of diseases, namely PD, inflammatory bowel disease, asthma, rheumatoid arthritis and others. Some proteins are involved in the inflammatory response, highlighting the interleukin network and some pathways like the T cell receptor signaling pathway and the Janus kinase/signal transducers and activators of transcription (Jak-STAT) signaling pathway. The JAK/STAT pathway is the main signaling mechanism for a wide array of cytokines and growth factors used to transduce a multitude of signals for development and homeostasis. JAK activation stimulates cell proliferation, differentiation, cell migration and apoptosis. The cellular events are critical to hematopoiesis, immune development, sexually dimorphic growth and other processes. Mutations that constitutively activate or fail to regulate JAK signaling properly cause inflammatory disease and leukemia [166]. The JAK/STAT pathway implies an inflammatory base, in this case the PD. This pathology is associated with elevated levels of cytokines that activate the JAK/STAT pathway [167]. A study indicates that the JAK/STAT pathway is inappropriately activated in

PD, which leads to neuroinflammation and neuronal damage. The inhibition of this pathway will be of benefit to PD patients [167].

The network of Imune system represented on ClueGO+CluePedia was also made **(Figure 23)**.

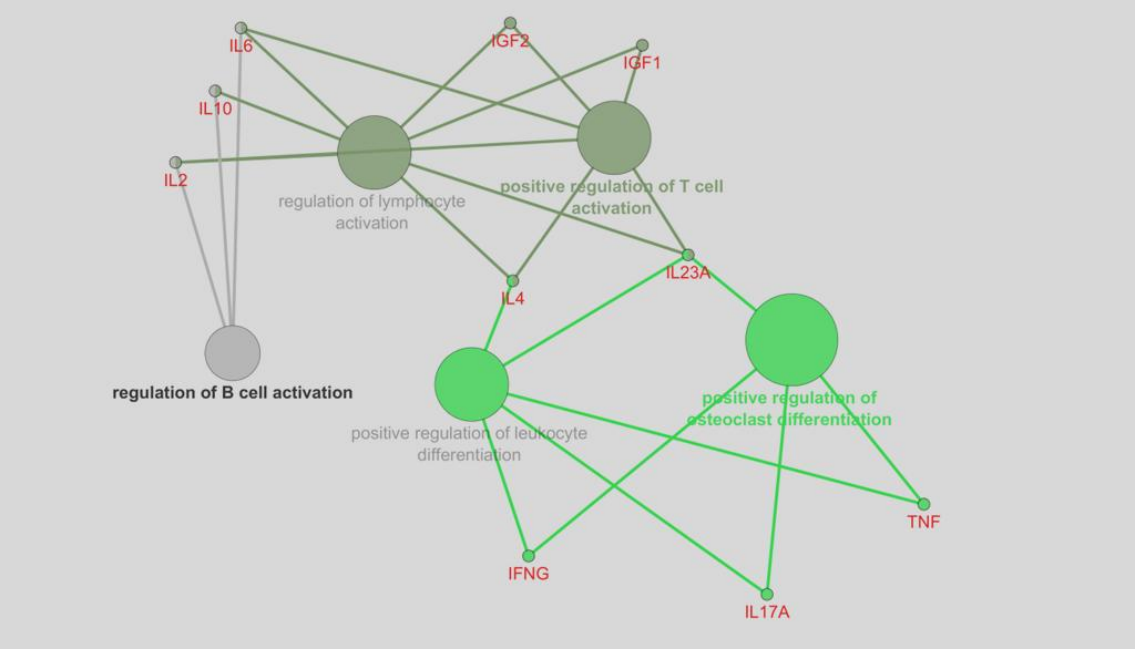


Figure 23- Network of Imune system represented on CluePedia. The small circles represents the proteins and the bigger the processes in which the proteins are involved. The lines represent the different interactions. Image generated by Cytoscape (ClueGO+CluePedia) on June 22, 2015.

In this network, we see the involvement of the proteins in the immune response in a greater detail. We see the interleukin network, and the proteins IL-2, IL-10, interferon G, IL-17, IL-4 and TNF present in this network are the same proteins found in the allograft rejection. With this information, it is possible that the inflammatory process lead to an auto immune response in ADHD.

4.3. Quantitative assessment of CCL13 and CCL3 in saliva samples from ADHD, dyslexic and healthy individuals

Once the saliva SOP was established a quantitative assessment of the expression of two chemokines associated with inflammation quantified in saliva from patients with ADHD, dyslexia and ADHD with dyslexia was done.

First, the protein concentration, volume and pH of saliva samples of healthy, hyperactive, dyslexic and hyperactive individuals with dyslexia were determined (27 samples in total). The hyperactivity, healthy, dyslexia and hyperactivity with dyslexia groups were compared in terms of total volume **(Figure 24A)**, protein concentration **(Figure 24B)** and pH **(Figure 24C).** There is no statistical significance between samples.

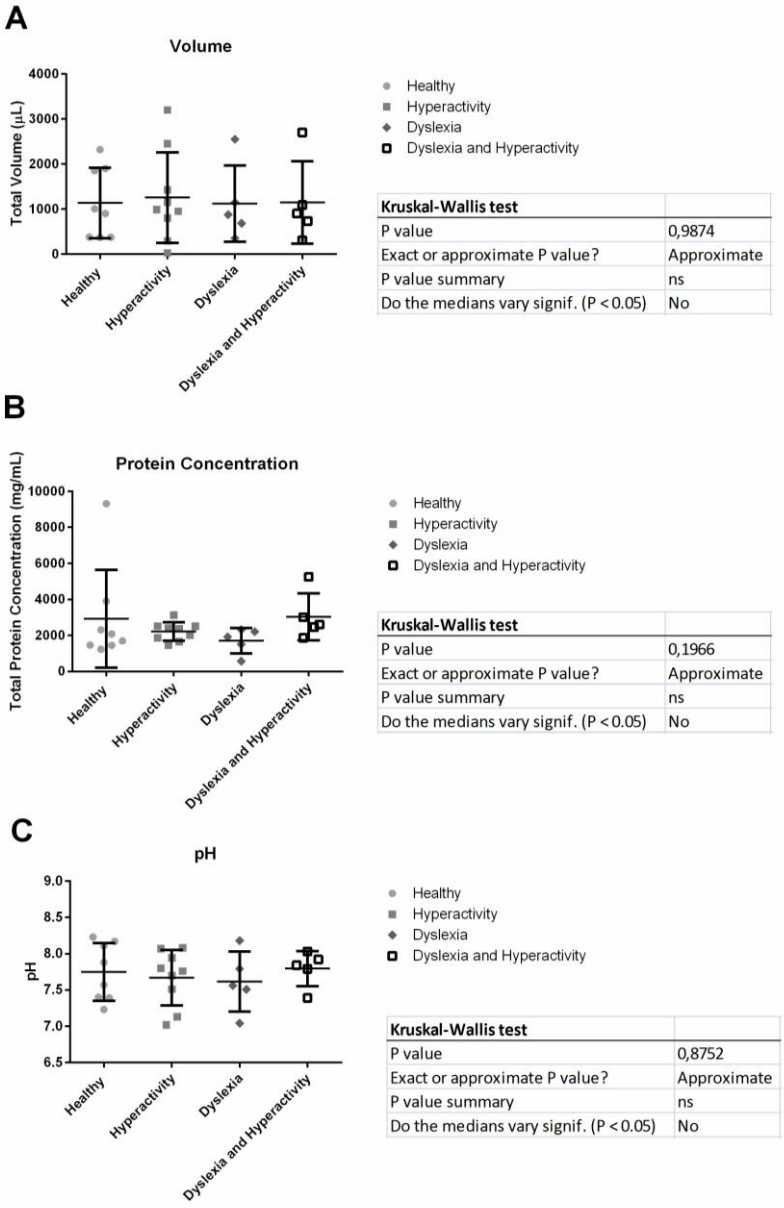


Figure 24- Comparison between the four groups considering the Volume of saliva collected (A), Protein Concentration (B) and pH (C). Statistical significance was determined by Kruskal-Wallis test of one-Way ANOVA. Data from 27 subjects.

The protein profiles of individuals with hyperactivity, dyslexia, hyperactivity with dyslexia and healthy were compared. For that, we determined the protein profiles of the 27 individuals. The results from the capillary electrophoresis are shown on Figure 25: protein electrophoretic profile of healthy individuals **(Figure 25, lanes 1-8)**, protein electrophoretic profile of dyslexic individuals **(Figure 25, lanes 9-13),** protein electrophoretic profile of hyperactive individuals with dyslexia **(Figure 25, lanes 14-18)** and protein electrophoretic profile of hyperactive individuals **(Figure 25, lanes 19-27).**

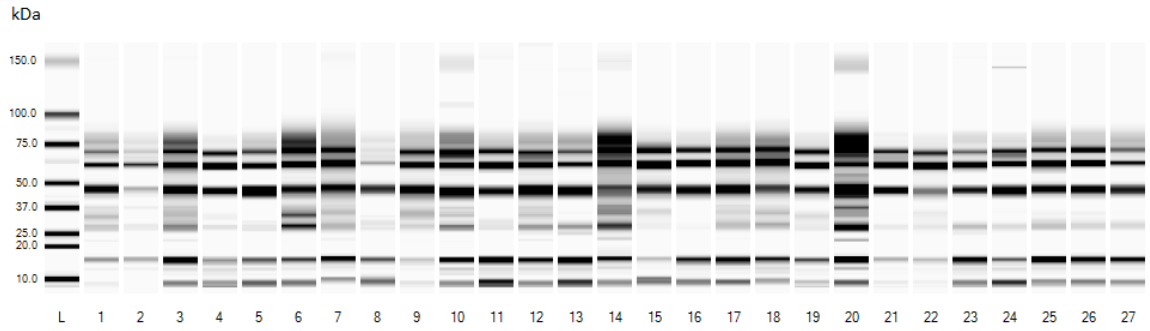


Figure 25- Protein profile from twenty seven saliva samples donors analysed by capillary electrophoresis using the Experion BioRad System. The protein electrophoretic profile is shown: healthy individuals (lanes 1-8), dyslexic individuals (lanes 9-13), hyperactive individuals with dyslexia (lanes 14-18) and hyperactive individuals (lanes 19-27). Ladder (L) with a range from 10 to 150 kDa.

In order to identify groups of profiles that may correspond to each clinical condition, the electrophoretic profile of the 27 samples is shown on **Figure 26** according to the MW and protein concentration. This analysis, and probably related to the reduced number of available samples, shows that there is no typical profile of the clinical situations. However by examining the most common profiles of each of the four groups there are differences visible in the profiles and it seems that there is a slight difference between the typical profiles of each group.

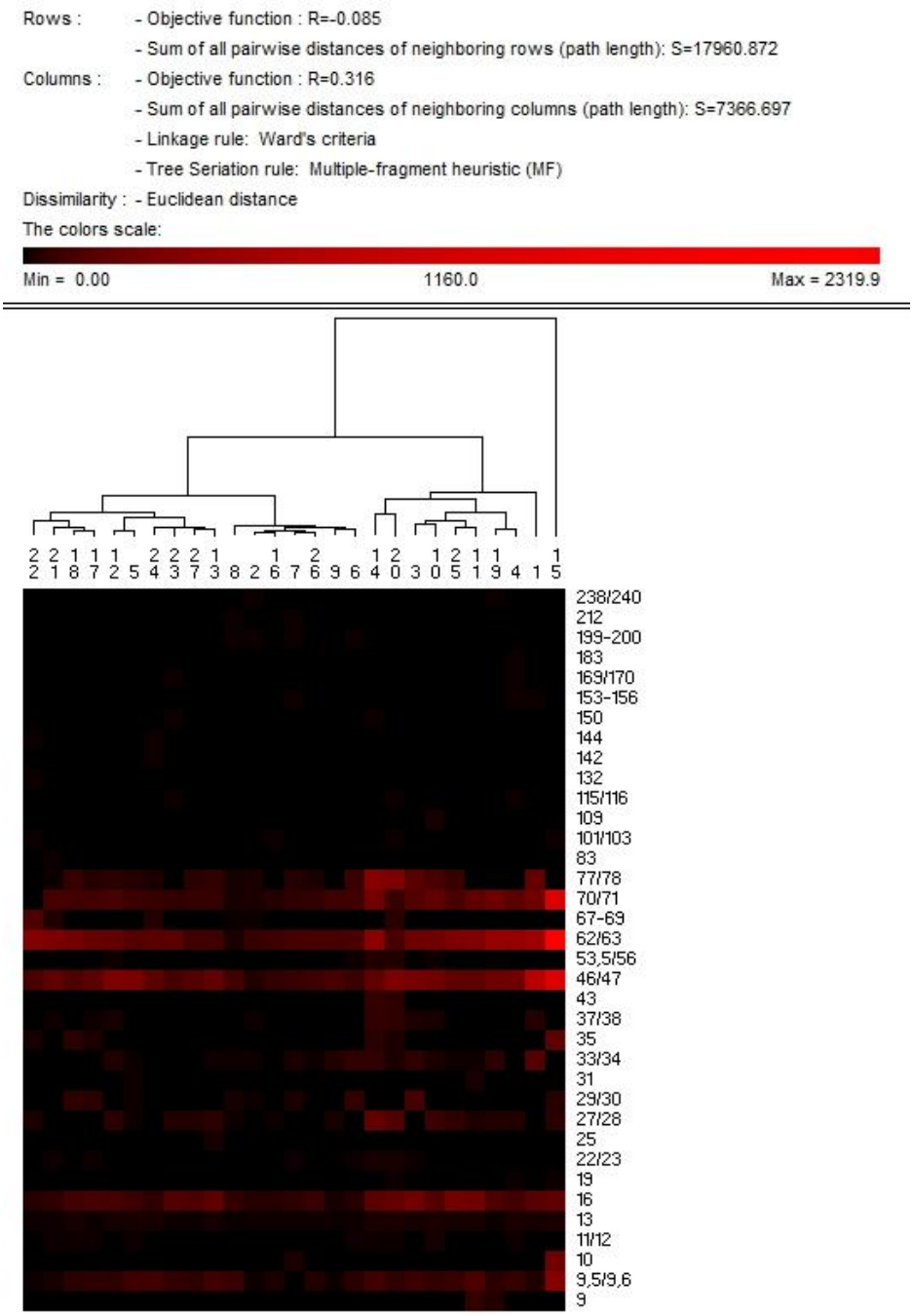


Figure 26- Heatmap visualizing the level of 36 bands differentially represented between subjects. Each column represents the data of protein concentration for all the subjects. Individuals 1- 8 are healthy, individuals 9-13 are dyslexic, individuals 14-18 are hyperactive with dyslexia and individuals 19-27 are hyperactive. Rows represent the different MW. The color code is graduated from black (under-representation compared to the group mean) to grey (over-representation compared to the group mean). Hierarchical clustering analysis was used to organize the map. The Cluster was done with the PermutMatrix program using the Ward's minimum variance method (n=27).

Protein concentration for each MW (protein band) in different individuals was compared using a non-parametric Kruskal-Wallis test of one-way ANOVA. These results, from the non-parametric Kruskal-Wallis test of one-way ANOVA show that the proteins with the molecular weights of 30, 35 and 200kDa) are significantly different ($p<0.05$) between individuals **(Annex 5)**. To see what are the differences identified previously by the Kruskal Wallis test, the Dunn's Multiple Comparison test of one-Way ANOVA **(Table 2)** comparing the mean of the three groups (hyperactivity, dyslexia and hyperactivity with dyslexia group) with the mean of the control group (healthy group) shows that there are significant differences between the healthy and the hyperactivity with dyslexia group in the MW of 35 kDa and 200 kDa ($p<0.05$). Between the healthy and the hyperactivity group there are also statistically significant differences in the bands with 30kDa and 200 kDa ($p<0.05$). Between the healthy and dyslexia group there is also statistical differences in the bands with 30kDa and 200 kDa ($p<0.05$).

Table 2- Comparison between the four groups (healthy, hyperactivity, dyslexia and hyperactivity with dyslexia group) in terms of protein concentration correspondent to each band of the total protein profile obtained by capillary electrophoresis.

	Dunn's multiple comparisons test	Significant?	Summary	Adjusted P Value
30 kDa	Healthy vs. Hiperactivity	Yes	**	0,0032
	Healthy vs. Dyslexia	Yes	*	0,0145
	Healthy vs. Dyslexia and Hiperactivity	No	ns	> 0,9999
	Dunn's multiple comparisons test	**Significant?**	**Summary**	**Adjusted P Value**
35 kDa	Healthy vs. Hiperactivity	No	ns	> 0,9999
	Healthy vs. Dyslexia	No	ns	> 0,9999
	Healthy vs. Dyslexia and Hiperactivity	Yes	**	0,0016
	Dunn's multiple comparisons test	**Significant?**	**Summary**	**Adjusted P Value**
200 kDa	Healthy vs. Hiperactivity	Yes	*	0,0139
	Healthy vs. Dyslexia	Yes	*	0,0475
	Healthy vs. Dyslexia and Hiperactivity	Yes	*	0,0475

With this analysis, we concluded that the bands with the MW 30, 35 and 200 kDa are the most variable between the different clinical situations. In the multicompromised individuals the bands with 35 and 200 are the more variables. Between all the groups the most variable are the band with 200 kDa. This implies

that the proteins localized in these MW bands are the most different between the clinical situations. The proteins identified by MS whose molecular weight is close to the proteins of interest (30, 35 and 200 kDa) are shown in **table 3**.

Table 3- Proteins identified by Mass Spectrometry analysis, according to the MW and Uniprot code.

	M.W. (kDa)	UniProt code	Protein name
Range from 30 to 35 kDa	28	P31947	14-3-3 protein sigma
	29	P06870	Kallikrein-1
	29	P18669	Phosphoglycerate mutase
	32	Q96DR5	Parotid secretory protein- PSP
	34	P25311	Zinc-alpha-2-glycoprotein (Zn-alpha-2-GP)
	35	P23280	Carbonic anhydrase 6
	36	P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
	36	P01857	Ig gamma-1 chain C region
	37	P01877	Ig alpha-2 chain C region
	37	P00338	L-lactate dehydrogenase A chain (LDH-A)
Range of 200 kDa	161	A8K2U0	Alpha-2-macroglobulin-like protein 1
	189	P01024	Complement C3-fragment

After the analysis of proteins and corresponding bibliography, we conclude that these conditions have an underlying inflammatory response. Therefore, we analyzed two chemokines involved in inflammation. Two saliva chemokines (CCL3 and CCL13) were quantified in 27 saliva samples of healthy, hyperactive, dyslexic and hyperactive children with dyslexia. Using the multiplex technique we are able to quantify the two chemokines on saliva samples.

Results show significant differences between the protein concentration of samples from the 4 groups under study (Kruskal Wallis test for protein concentration of CCL3, $p<0.05$ and CCL13, $p<0.05$; **Figures 27 A and B**). Then, a Dunn's multiple comparison test was done to understand these differences. CCL3 is significantly increased in the samples from the hyperactive group with dyslexia (when compared to the control group; $p<0.01$; **Figure 27A)**. The same trend was found for CCL13: increase in the samples from the hyperactive group with dyslexia ($p<0.05$; **Figure 27B)**.

These results show that CCL3 and CCL13 are increased only in the multi compromised group. Despite not having a sufficient number of samples for a solid statistical treatment, we see that the hyperactive group has the levels of these two cytokines increased nearly five times. There is an increase in the protein concentration in both proteins from 10 pg/mL in the healthy group to 50 pg/mL in the hyperactive group.

This work shows for the first time that it is possible to evaluate these two chemokines, CCL3 and CCL13 in saliva, whereas others authors report zero values for CCL3 on saliva samples using Multiplex technology [168].

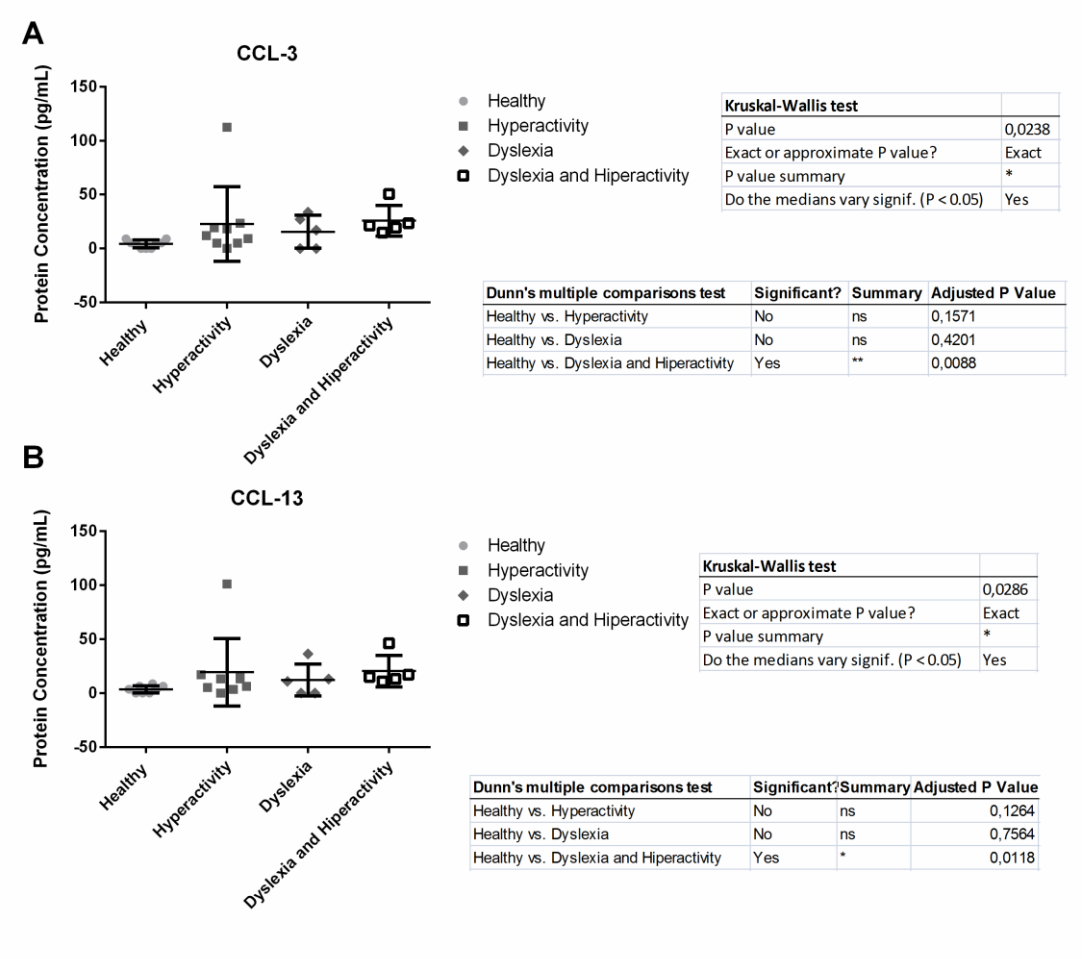


Figure 27- Comparison between the four groups considering the protein concentration of CCL3 (A) and the protein concentration of CCL13 (B). Statistical significance was determined by Dunnett's Multiple Comparison test of one-Way ANOVA.

The pro inflammatory chemokines CCL3 and CCL13 were quantified in saliva due to the fact that these proteins are increased in psychiatric and neurological disorders such as AD, epilepsy and autoimmune encephalomyelitis.

CCL13, during chronic inflammation, is expressed in nonlymphoid tissues and acts as a chemotactic factor to attract monocytes in tissues exposed to exogenous pathogens [169]. CCL3 plays a role in recruiting macrophages, dendritic cells and T cells to site of infection and lymphoid organs [170] Previous studies suggest that this chemokine levels are associated with a stronger Th1 response [171] and has been implicated in suppression of hematopoietic stem and progenitor cell proliferation [172]

The children that participate in our study manifest inflammation since the values of these two chemokines are increased which can indicate that neuroinflammation can be present in these pathologies.

5. Conclusion

The saliva sample collection brings together a number of advantageous characteristics that allows us evaluate the pathologies by molecular biology methods, like electrophoresis, western blot, Enzyme-Linked Immunosorbent Assay and multiplex technology. We created a protocol for collection of whole saliva based on cotton-roll placed sublingually that is easy and inexpensive and produces samples suitable for protein analysis. The samples have sufficient quality and protein concentration to be stored by the Universidade Católica Portuguesa and associated IMM Biobank. We also were able to demonstrate that the SOP and the multiplex technique are established for these samples of ADHD and dyslexia. This technology and protocol can be used for other pathologies using a small quantity of saliva samples.

Further research and validation studies of other possible inflammatory markers are needed for a better comprehension of these pathologies.

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7. Annexes

Are available as annexes the informed consent **(Annex 1)** and questionnaire **(Annex 2)** given to donors before the collection of saliva samples, the protocol of sublingual saliva collection, processing and storage **(Annex 3)**, a table with the list of the reviewed proteins identified in neuropsychiatric disorders **(Annex 4)** and the table with the Kruskal-Wallis statistical analysis **(Annex 5)**.

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7.1 Annex 1: Informed Consent

UNIVERSIDADE CATÓLICA PORTUGUESA

salivaTec - UCP
Laboratório de Investigação Interdisciplinar em Saliva

DECLARAÇÃO DE CONSENTIMENTO INFORMADO

INFORMAÇÃO AO DADOR DE AMOSTRAS BIOLÓGICAS

Título do projeto de investigação

Projeto de investigação que irá decorrer no SalivaTec (Laboratório de Investigação Interdisciplinar em Saliva) do Departamento de Ciências da Saúde da Universidade Católica Portuguesa.

Objetivo do Estudo
A recolha de amostras biológicas humanas e seu posterior armazenamento no Biobanco de amostras do Instituto de Medicina Molecular (IMM) da Faculdade de Medicina da Universidade de Lisboa, permitirá a realização de investigação para o esclarecimento a nível molecular de doenças e para o desenvolvimento de diagnóstico e em múltiplas áreas da Saúde. Contudo, este objetivo só será cumprido com a colaboração dos doentes e de indivíduos saudáveis, através da doação de amostras biológicas que serão guardadas e preservadas em condições apropriadas de forma a serem utilizadas para futuros estudos. Caso o doente ou indivíduo saudável e/ou o seu representante legal decida participar, terá de fazer apenas os procedimentos habituais de uma consulta.

Procedimentos
No caso de concordar em participar neste projeto, ser-lhe-á colhida uma amostra biológica. A amostra habitualmente solicitada será realizada a partir da colheita de saliva. Para os indivíduos que estejam a realizar exames diagnósticos ou que estejam a ser sujeitos a tratamentos cirúrgicos poderá ser pedida autorização para colheita de uma pequena amostra do material removido durante o procedimento (como por exemplo tecidos removidos para biópsias ou removidos no decurso de cirurgias). Estas colheitas serão efetuadas sem alterar os procedimentos médicos habituais e sem interferir com a rentabilidade diagnóstica do procedimento ou com o sucesso da cirurgia. Esta amostra será preservada em condições apropriadas e as informações clínicas com ela relacionada serão introduzidas numa base de dados, passando a sua identificação pessoal a estar codificada e não acessível aos utilizadores das amostras.
A doação da amostra é voluntária e revogável, sendo que o dador, ou o seu representante legal, tem o direito de retirar a amostra e/ou interromper a colaboração assim que achar conveniente, sem necessidade de justificação e não podendo ser descriminado por isso. O dador ou o seu representante legal deverá manifestar por escrito a sua vontade em retirar a amostra ou interromper a colaboração e nestas situações a amostra será imediatamente destruída.
O SalivaTec propõe-se armazenar as amostras biológicas e seus possíveis derivados tais como DNA e RNA nas instalações do Biobanco do Instituto de Medicina Molecular. O SalivaTec não divulgará resultados envolvendo o material biológico. No entanto, o dador poderá escolher se quer ser informado dos resultados com potencial relevância para a sua saúde. O pedido de resultados deverá ser feito por escrito para o SalivaTec pelo dador ou representante legal e deve ser expresso no consentimento informado.

Serão cumpridas todas as normas éticas aceites internacionalmente para o uso de matérias biológicos para fins de investigação. Todos os projetos que fizerem uso das amostras depositadas no Biobanco serão submetidos à Comissão de Ética competente para a sua avaliação.

Identificação das amostras e Confidencialidade
A existência de um Biobanco pressupõe a existência de uma base de dados contendo informação clínica referente ao doente ou indivíduo saudável. Após a colheita, as amostras serão identificadas por um código de forma a preservar a privacidade.
Durante o desenvolvimento de um projeto de investigação, a equipa de investigação poderá ter necessidade de recolher informação do processo clínico para a execução do estudo. O anonimato será, contudo mantido, ou seja os dados constantes do seu processo clínico serão fornecidos ao investigador, mas sem qualquer identificação, ou qualquer informação que permita saber a quem pertencem.
A descodificação apenas poderá ser efetuada pelo médico (que será o responsável pela base de dados, de acordo com a informação fornecida à Comissão Nacional de Proteção de Dados - CNPD), em caso de absoluta

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DECLARAÇÃO DE CONSENTIMENTO INFORMADO

necessidade, por motivos de saúde do dador e, a pedido deste, e sempre de acordo com as disposições legais em vigor.
Os dados serão tratados confidencialmente, de acordo com a Lei, com os regulamentos e de acordo com as normas éticas aprovadas pela Comissão de Ética da Universidade Católica Portuguesa.
Os dados resultantes dos estudos realizados serão alvo de publicação de uma forma anónima e agregada, em termos de percentagens ou de dados numéricos, nunca individualmente.

Tempo de conservação
As amostras serão conservadas por um período de 20 anos no Biobanco do Instituto de Medicina Molecular (IMM), sob a responsabilidade da Equipa ligada ao SalivaTec, enquanto este estiver devidamente credenciado pelas entidades competentes. As coleções de amostras serão avaliadas periodicamente, nomeadamente para aferir da sua qualidade, podendo ser destruídas ou, findo o período da conservação, poder-se-á solicitar a prorrogação da conservação. Nestas condições excecionais o SalivaTec poderá voltar a contactar os dadores.

Comunicação e divulgação de dados
Os dados genéticos e as amostras biológicas colhidas para fins de investigação científica podem ser transferidos para outras organizações ou centros de investigação, para fins de pesquisa e somente em projetos desenvolvidos conjuntamente com o SalivaTec, mediante consentimento do participante expresso na declaração de consentimento informado.

Possíveis Benefícios para os Participantes
Esta é uma doação altruísta, não havendo por isso qualquer compensação para o dador. Não se garante que este estudo envolva quaisquer benefícios diretos para o participante. Se algum dos estudos puder ser relevante para a saúde do dador, este será informado, se essa for a sua vontade expressa na declaração de consentimento informado. Contudo, a sua participação proporcionará a aquisição de conhecimentos que poderão vir a beneficiá-lo a si ou a terceiros no futuro.

Riscos físicos previsíveis
Na maioria dos casos, os riscos e o desconforto associados serão mínimos ou inexistentes. Nas colheitas associadas a procedimentos com fins diagnósticos ou terapêuticos, os riscos e o desconforto serão os inerentes ao procedimento em si. Em qualquer dos casos, o dador será sempre antecipadamente informado dos riscos e grau de desconforto associados aos procedimentos.

Participação Voluntária e Direitos de Abandono
O presumível dador terá toda a liberdade para se recusar a participar no estudo ou retirar o seu consentimento, suspendendo a participação em qualquer momento e, consequentemente, as amostras serão destruídas. A participação é voluntária e a sua recusa em participar não envolverá qualquer penalização ou perda de benefícios. A recusa ou abandono não colocarão em risco o direito a receber tratamento ou assistência médica, presentemente ou no futuro.
O dador poderá retirar o seu consentimento nas modalidades **sem contacto futuro** (as amostras poderão ser usadas normalmente até se esgotarem, mas não serão estabelecidos futuros contactos para a obtenção de mais amostras) ou **sem uso futuro** (não serão estabelecidos futuros contactos e as amostras serão imediatamente destruídas e os registos eliminados).

Se tiver qualquer dúvida, em qualquer momento, mesmo após a colheita, sobre este estudo poderá contactar a Diretora do SalivaTec: Prof. Doutora Marlene Barros, dirigindo-se a:
SalivaTec
Departamento de Ciências da Saúde
Universidade Católica Portuguesa
Tel. +351232419500 - Fax +351232428344
E-mail: mbarros@crb.ucp.pt

DECLARAÇÃO DE CONSENTIMENTO INFORMADO

DECLARAÇÃO DE CONSENTIMENTO INFORMADO

Banco de amostras biológicas para fins de investigação biomédica

Investigador:_____ Local de recolha: _____
Nome do dador: _____
Número de estudo do dador: _____

Eu, , portador do bilhete de identidade/cartão do cidadão n.º [ ], declaro ter tomado conhecimento e aceitar participar neste projeto, de forma a contribuir para a criação de um banco de amostras biológicas com informação clínica associada, para fins de investigação biomédica.
Aceito que a minha amostra biológica seja utilizada em projetos de investigação de mecanismos das doenças, diagnóstico precoce, fatores de prognóstico e novos alvos terapêuticos em múltiplas áreas da medicina. Poderei revogar a autorização para utilização da minha amostra biológica e informação clínica em qualquer altura. O objetivo do banco de amostras biológicas foi-me claramente explicado e foi-me dada a oportunidade de colocar questões sobre o seu funcionamento, bem como os procedimentos relativos à colheita e utilização da minha amostra biológica e dados a ela associados.
Declaro que aceito participar, voluntariamente, neste estudo. Especificamente concordo com os seguintes pontos:

- Consinto a colheita de material biológico (saliva / ..... / .....) e autorizo a conservação de amostras no Biobanco, de modo a que possam ser usados para pesquisas futuras, incluindo estudos genéticos por investigadores portugueses e estrangeiros, sem fins lucrativos;

Sim ☐ Não ☐

- **Esta opção é para ser respondida apenas por participantes que já cederam amostras biológicas colhidas no âmbito de outros projetos.** Nestas circunstâncias, autorizo a transferência para o Biobanco das minhas amostras biológicas, previamente colhidas no âmbito de outros projetos, de modo que elas possam ser utilizadas em pesquisas futuras, incluindo estudos genéticos por investigadores portugueses e estrangeiros, mas sem fins lucrativos;

Sim ☐ Não ☐

- Estou consciente de que a minha participação é voluntária e que posso em qualquer altura solicitar a destruição das minhas amostras biológicas, invalidando assim o consentimento informado prévio, sem justificar, tendo recebido a garantia de que o meu pedido não desenvolverá discriminação;

Sim ☐ Não ☐

- Declaro que quero conhecer resultados que possam ser relevantes para a minha saúde.

Sim ☐ Não ☐

DECLARAÇÃO DE CONSENTIMENTO INFORMADO

I

- Autorizo ser contactado novamente pelo Biobanco para pedido de atualização sobre a minha situação clínica;

Sim ☐ Não

- Autorizo o contactado do Biobanco a familiares meus para pedido autorização de colheita de amostras biológicas e/ou informação clínica;

Sim ☐ Não ☐

_____ _____

Data Assinatura do Dador/Representante Legal

Em caso de representante legal, este atua na qualidade de:

□ Titular do poder paternal, quando o dador é menor

□ Tutor, quando o dador foi declarado interdito

□ Herdeiro, quando o dador faleceu

Discuti este estudo de investigação com o participante e/ou o seu representante legal, utilizando uma linguagem compreensível e apropriada. Informei adequadamente o participante sobre a natureza deste estudo e sobre os seus possíveis benefícios e riscos, considerando que o participante compreendeu a minha explicação.

_____ _____ _____

Data Nome do Investigador/ Médico Assinatura do Investigador/ Médico

Foi entregue um duplicado deste documento ao doente/representante legal.

7.2 Annex 2: Donors Questionnaire

Código do Dador:
Dador nº:
NP:

Questionário de Dadores

1. ***Informação geral acerca do dador***

1.1 Data nascimento: ____/____/____

1.2 Género:

❒Masculino ❒Feminino

1.3 Dados Biométricos:

❒**Altura:** _____ Cm ❒**Peso:** _____ Kg ❒**Perímetro Abdominal:** _____ cm

1.4 Etnia:

❒Caucasiana ❒Negra ❒Asiática ❒Cigana

❒Outra. Qual?

_____

1.5 Área de residência:

❒Aldeia ❒ Vila ❒Cidade

1.6 Estado civil:

❒Solteiro ❒Casado ❒Vive maritalmente ❒Viúvo ❒ Divorciado

1.7 Nível de escolaridade:

❒Básico (abaixo do 9º Ano) ❒Médio (12º Ano)

❒Licenciatura, Mestrado e/ou Doutoramento

1.8 Profissão:_____

Código do Dador:
Dador nº:
NP:

2. *Hábitos Tabágicos*

2.1 Fuma ou já fumou?

❒Não ❒Sim

❒Ex-fumador. **Há quantos anos deixou de fumar?** _____ Anos

2.2 Se sim:

2.2.1 Com que idade começou a fumar: _____ anos

2.2.2 Quantos cigarros fuma por dia: _____ cigarros

3. *Consumo de Álcool*

3.1 Bebe bebidas alcoólicas?

❒Não Passe para a questão 3.4 ❒Sim.

Com que idade começou a beber: _____ anos

3.2 Frequência do consumo de álcool Nº copos de vinho/semana _____ Nº cervejas/semana _____

Nº de digestivos/semana) _____

3.3 Deixou de beber?

❒Não ❒Sim. **Há quantos anos deixou de beber?** _____ anos.

4. *Exercício Físico*

Questionário de Baecke para Avaliação da actividade física habitual em adultos (1) (Adaptado) *Por favor, circule a resposta apropriada para cada questão pensando nos últimos 12 meses*		
1. Você pratica ou praticou desporto ou exercício físico nos últimos 12 meses:	Sim	Não
1.1 Qual desporto ou exercício físico pratica ou praticou mais frequentemente?		
1.1.1 Quantas horas por semana?		
1.1.2 Quantos meses por ano?		
1.2 Se pratica ou praticou outro desporto ou exercício físico além do anterior, qual o tipo?	Sim	Não

1.2.1 Quantas horas por semana?					
1.2.2 Quantos meses por ano?					
	1	2	3	4	5
2. Em comparação com outros da minha idade, eu penso que minha atividade física durante as horas de lazer é:	Muito menor	Menor	A mesma	Maior	Muito maior
3. Durante as horas de lazer eu suo:	Nunca	Raramente	Algumas vezes	Frequentemente	Muito frequentemente
4- Durante as horas de lazer eu pratico desporto ou exercício físico:	Nunca	Raramente	Algumas vezes	Frequentemente	Muito frequentemente
5. Durante as horas de lazer eu vejo televisão:	Nunca	Raramente	Algumas vezes	Frequentemente	Muito frequentemente
6. Durante as horas de lazer eu ando:	Nunca	Raramente	Algumas vezes	Frequentemente	Muito frequentemente
7. Durante as horas de lazer eu ando de bicicleta:	Nunca	Raramente	Algumas vezes	Frequentemente	Muito frequentemente
8. Durante quantos minutos por dia você anda a pé ou de bicicleta indo e voltando do trabalho, escola ou compras? Total de minutos:_____	< 5	5-15	16-30	31-45	> 45

1-Florindo A, Latorre M. Validação e reprodutibilidade do questionário de Baecke de avaliação da atividade física habitual em homens adultos. Rev Bras Med Esporte [Internet]. 2003 [cited 2014 Dec 15];9(11):121–8. Available from: http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Validação+e+reprodutibilidade+do+questionário+de+Baecke+de+avaliação+da+atividade+física+habitual+em+homens+adultos+*#0

Código do Dador:
Dador nº:
NP:

5. Hábitos Alimentares

	Nunca (0 vezes/ semana)	Raramente (1 a 2 vezes/ semana)	Algumas vezes (3 a 4 vezes/ semana)	Muitas vezes (5 a 6 vezes/ semana)	Sempre (7 ou mais vezes/ semana)
Após acordar tenho por hábito tomar o café da manhã.					
Como e mastigo devagar.					
Passo muitas horas sem comer.					
Faço 5 ou 6 refeições por dia.					
Faço refeições com intervalos de 3 a 4 horas.					
O jejum nocturno não ultrapassa as 10 horas.					
Ingiro leite/iogurte/queijo.					
Como fruta.					
Como legumes e hortaliças.					
Como sopa.					
Como alimentos integrais ricos em fibra.					
Como carnes gordas.					
Como carnes magras.					
Adiciono aos alimentos produtos industriais.					
O azeite faz parte da minha alimentação.					
Consumo molhos gordos.					
Como pizzas, hambúrgueres e cachorros-quentes.					
Consumo produtos de charcutaria.					
Prefiro a comida com um pouco de sal a mais.					
Ingiro alimentos salgados.					
Ingiro alimentos ricos em açucar.					
No lanche, como um bolo ou um salgado.					
Quando como um doce opto por fazê-lo...					
Prefiro comer bolos/bolachas do que comer pão.					
Utilizo bastante açúcar para adoçar.					
Adopto uma alimentação variada às refeições.					
Faço uma refeição de peixe.					
Faço uma alimentação à base de cozidos e grelhados.					
Costumo comer as partes queimadas dos alimentos.					
Como alimentos fritos e assados no forno.					
Faço refeições abundantes.					

Petisco entre refeições.					
Como quase sempre o mesmo tipo de alimentos.					
Consumo bebidas alcoolicas fora das refeições.					
Só bebo água quando tenho sede.					
Bebo pelo menos 1,5 L de água por dia.					
Consumo alimentos pré-cozinhados e enlatados.					
Como pão de mistura tipo caseiro.					
Como arroz/massa e batata.					
Como peixes gordos.					

Marques A, Luzio F, Martins J, Vaquinhas M. Hábitos alimentares: Validação de uma escala para a população portuguesa. Esc Anna Nery. 2011;15(2): 402-9.

6. Saúde

6.1 Grupo Sanguíneo

❒A ❒B ❒AB ❒O

❒Rh+ ❒Rh-

❒Não sabe

6.2 Toma regularmente medicamentos?

❒Não ❒Sim

6.3 Indique todos os medicamentos que toma regularmente:

Medicação (DCI)	mg	Há quantos anos começou

6.4 Tomou alguma medicação que não seja a indicada na tabela acima referida nos últimos 30 dias?

❒Não ❒Sim. **Qual?**

_____

6.5 Tomou algum antibiótico nos últimos 3 meses?

❒Não ❒Sim. **Qual?**

_____

6.6 Tomou corticosteroides nos últimos 30 dias?

❒Não ❒Sim

6.7 Tomou bifosfonatos nos últimos 30 dias?

❒Não ❒Sim

6.8 Tem as vacinas em dia?

❒Não ❒Sim

→ Se é homem passe para a questão 6.13.

6.9 Está grávida?

❒Não ❒Sim. **De quantos meses?** _____ meses.

6.10 Encontra-se na menopausa?

❒Não ❒Sim. **Há quanto tempo?** _____ meses.

6.11 Há quanto tempo teve a última menstruação? _____ dias.

6.12 Toma anticoncecionais?

❒Não ❒Sim. **Qual?**

_____

6.13 Nos últimos 12 meses foi consultado por um médico?

❒Não ❒Sim

6.14 Se sim, que especialidade? (Pode selecionar mais do que uma opção)

❒Médico de consulta geral e familiar ❒Dermatologista

❒Gastroenterologia ❒Ortopedista

❒Outro (s)

_____

6.15 Qual a data das últimas análises que efetuou? ____/____/____

6.16 Foram encontrados valores anormais?

❒Não ❒Sim. **Quais?**

_____

6.17 Tem hipertensão?

❒Não ❒Sim

6.18 Atualmente sofre de alguma enfermidade?

❒Não ❒Sim

6.19 Se sim, qual? (Pode selecionar mais do que uma opção)

❒Problemas cardíacos:

❒Doença das artérias coronárias ❒Ataque cardíaco

❒Angina ❒Aneurisma da aorta

❒Arritmias ❒Doença cardíaca congénita

❒Insuficiência cardíaca ❒Doença cardíaca reumática

❒Outro(s)

_____

❒Diabetes:

❒Tipo 1 ❒Tipo 2

Análise Clínica	Valores
Colesterol	
Glicose	
Resistência à insulina	
Hemoglobina glicosilada	
AGEs	

❒ Doenças auto-imunes:

❒ Doença de Crohn ❒ Doença de Behçet

❒ Doença de Graves ❒ Síndrome de Sjogren

❒ Outra:

_____

❒ Doenças de sangue. Quais?

_____

❒ Doenças infeto-contagiosas. Quais?

_____

❒ Doenças de fígado. Quais?

_____

❒ Problemas de estômago. Quais?

_____

❒ Problemas renais. Quais?

_____

❒ Epilepsia

❒ Asma

❒ Urticária

❒ Sinusite

❒ Acne

❒ Outra (s).

Quais?_____

6.20 Alergias

6.20.1 É alérgico a algum medicamento ou dispositivo médico?

❒ Não ❒ Sim. **Qual?**

_____

6.20.2 É alérgico a algum alimento?

❒ Não ❒ Sim. **Qual?**

_____

6.20.3 É alérgico a picadas de insetos?

❒ Não ❒ Sim. **Qual?**

_____

6.21 Foi sujeito a algum tratamento de radioterapia ou quimioterapia?

❒Não ❒Sim. **Há quanto tempo?** _____ meses.

6.22 História Familiar – Existem doenças na família como:

❒Doenças Cardíacas ❒Diabetes ❒Cancro ❒Não sabe

❒Outras, quais?_____

7. *Patologia do sono*

Nas últimas duas semanas:	Nunca	Raramente	Por vezes	Frequentemente	MPA	Sempre
adormeceu sentado e a ler						
adormeceu a ver tv						
adormeceu sentado em local público						
adormeceu como passageiro de um carro, uma hora sem intervalo						
adormeceu deitado para descansar após o almoço						
adormeceu sentado e a falar com alguém						
adormeceu sentado após o almoço sem ingerir álcool						
adormeceu a conduzir um carro em trânsito lento						
Ao deitar tem dificuldade de dormir?						
Acordou durante o sono e teve dificuldade para adormecer de novo?						
Acordou muito cedo e não conseguiu voltar a adormecer?						

8. *Escala de Felicidade Subjectiva (SHS)*

Desenvolvida por: Sonja Lyubomirsky.

Instruções: Para cada uma das seguintes frases, por favor, escolha o ponto da escala que o descreve de forma mais apropriada.

1 2 3 4 5 6 7

Uma pessoa não muito feliz	Uma pessoa muito feliz

1	Em geral, considero-me:	
2	Comparado com os meus pares, considero-me:	
3	Algumas pessoas são geralmente muito felizes. Apreciam a vida independentemente do que se passa à sua volta, aproveitando o melhor de tudo. Como considera essa pessoa?	
4	Algumas pessoas não são geralmente felizes. Embora não estejam deprimidas, nunca parecem tão felizes como poderiam ser. Como considera essa pessoa?	

7. *Escala de Satisfação com a Vida (SWLS)*

Desenvolvida por: Ed Diener

Instruções: Abaixo encontrará cinco afirmações com as quais pode ou não concordar. Usando a escala de resposta a seguir, que vai de 1 a 7, indique o quanto concorda ou discorda com cada uma; escreva um número no espaço ao lado da afirmação, segundo sua opinião. Por favor, seja o mais sincero possível nas suas respostas.

7 = Concordo totalmente
6 = Concordo
5 = Concordo ligeiramente
4 = Nem concordo nem discordo
3 = Discordo ligeiramente
2 = Discordo
1 = Discordo totalmente

1._____Na maioria dos aspectos, a minha vida é próxima ao meu ideal.
2._____As condições da minha vida são excelentes.
3._____Estou satisfeito(a) com minha vida.
4._____Dentro do possível, tenho conseguido as coisas importantes que quero da vida.
5._____Se pudesse viver uma segunda vez, não mudaria quase nada na minha vida.

Código do Dador:
Dador nº:
NP:

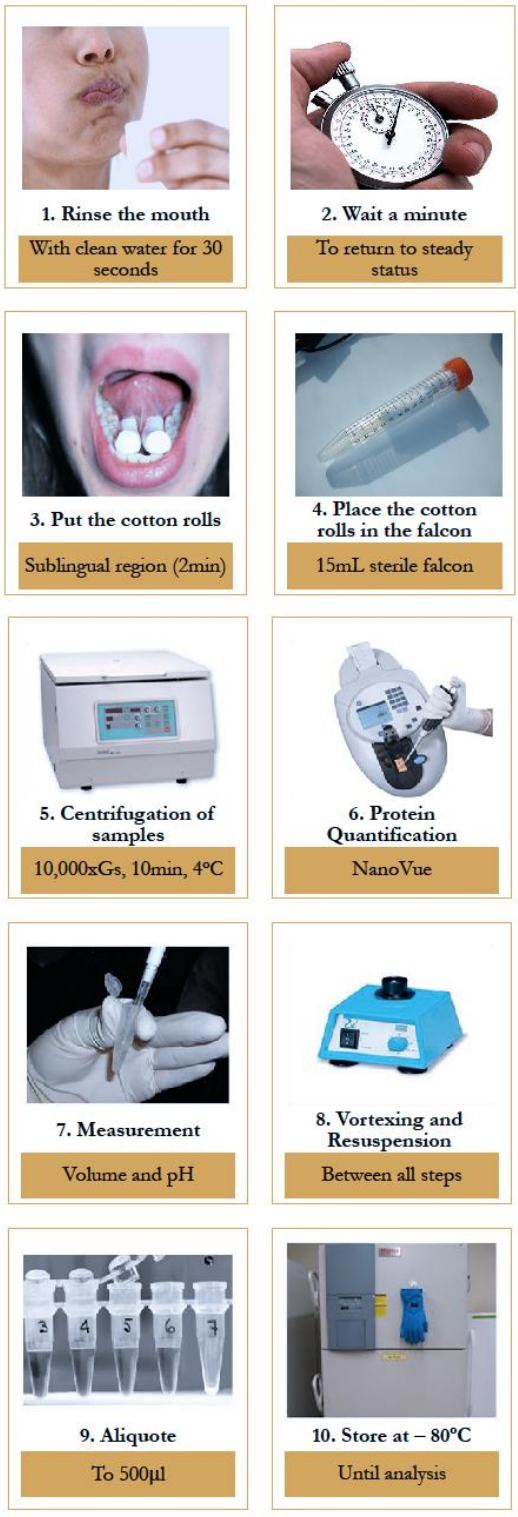
7. Questionário de Capacidades e Dificuldades

Instruções: Encontra a seguir 25 frases. Para cada uma delas marque, com uma cruz, um dos quadrados. Por favor, responda com base no comportamento do seu filho/a nos últimos 6 meses.

	Não é verdade	É um pouco verdade	É muito verdade
1. É sensível aos sentimentos dos outros	☐	☐	☐
2. É irrequieto/a, muito mexido/a, nunca pára quieto/a	☐	☐	☐
3. Queixa-se frequentemente de dores de cabeça, dores barriga ou vómitos	☐	☐	☐
4. Partilha facilmente com os outros adolescentes (as suas coisas, alimentos, etc.)	☐	☐	☐
5. Enerva-se muito facilmente e tem "crises de raiva"	☐	☐	☐
6. Tem tendência a isolar-se, gosta mais de estar sozinho/a (sem amigos ou família)	☐	☐	☐
7. Obedece com facilidade, faz habitualmente o que os adultos lhe mandam	☐	☐	☐
8. Tem muitas preocupações, parece sempre preocupado/a	☐	☐	☐
9. Gosta de ajudar se alguém está magoado, aborrecido ou doente	☐	☐	☐
10. Não sossega. Está sempre a mexer as pernas ou as mãos	☐	☐	☐
11. Tem pelo menos um bom amigo/ uma boa amiga	☐	☐	☐
12. Luta frequentemente, ameaça ou intimida os outros	☐	☐	☐
13. Anda muitas vezes triste, desanimado/a ou choroso/a	☐	☐	☐
14. Em geral os outros jovens gostam dele/a	☐	☐	☐
15. Distrai-se com facilidade, está sempre de cabeça no ar	☐	☐	☐
16. Em situações novas é receoso/a, muito agarrado/a e pouco seguro/a	☐	☐	☐
17. É simpático/a e amável com as crianças mais pequenas	☐	☐	☐
18. Mente frequentemente ou engana	☐	☐	☐
19. Os outros metem-se com ele/a, ameaçam-no/a ou intimidam-no/a	☐	☐	☐
20. Sempre pronto/a a ajudar (pais, professores, outros adolescentes)	☐	☐	☐
21. Pensa nas coisas antes de as fazer	☐	☐	☐
22. Rouba em casa, na escola e em outros sítios	☐	☐	☐
23. Dá-se melhor com os adultos do que com os adolescentes	☐	☐	☐
24. Tem muitos medos, assusta-se com facilidade	☐	☐	☐
25. Geralmente acaba o que começa, tem uma boa atenção	☐	☐	☐

Fonte: Goodman, 2001

7.3 Annex 3: Protocol for collection, processing and storing saliva samples



7.4 Annex 4: Review of the literature of proteins related to neuropsiquiatric diseases.

List with the 56 proteins, uniprotKBAC code (obtained by http://www.uniprot.org).

UniProtKBAC	Name	Organism	Whole Saliva	Brain tissue	CSF	Serum	Plasma	Blood	Health	Disease (OMIM ID)	Regulation	Age group	Gender*	Methods of Sampling**	Methods of Analysis***	Type of Study	Citation (NCBI ID)
P08582	Melanotranferrin	Homo sapiens (Human)	x						x	Alzheimer's Disease					Western Blot in SDS PAGE	non-proteomics	12809550
P05067	Beta-amyloid	Homo sapiens (Human)								Alzheimer's Disease							
Q6FI27	GSK3β	Homo sapiens (Human)								Alzheimer's Disease							
P37840	alpha-Synuclein	Homo sapiens (Human)	x						x	Parkinson's Disease		39-82	17M / 7F	unstimulated whole saliva sample	Western Blot, In-Gel digestion, Mass spectrometry, Immunoprecipitation,Luminex Assay	non-proteomics	21349902
Q99497	DJ-1	Homo sapiens (Human)	x						x	Parkinson's Disease		39-82	17M / 7F	unstimulated whole saliva sample	Western Blot, In-Gel digestion, Mass spectrometry, Immunoprecipitation,Luminex Assay	non-proteomics	21349902
P02649	apolipoprotein E	Homo sapiens (Human)	x						x	Parkinson's Disease		70-85	M/F		DNA Extraction, MassARRAY, genotyping	non-proteomics	21741729
Q17RV3	Leucine-Rich Repeat Kinase-2	Homo sapiens (Human)							x	Parkinson's Disease						Genomic Analysis	21238487
Q6S8G7	Parkin	Homo sapiens (Human)							x	Parkinson's Disease						Genomic Analysis	21238487
Q9NQ11	ATP13A2	Homo sapiens (Human)							x	Parkinson's Disease						Genomic Analysis	21238487
Q9BXM7	PINK-1	Homo sapiens (Human)							x	Parkinson's Disease						Genomic Analysis	21238487
P09936	UCH-L1	Homo							x	Parkinson's Disease						Genomic Analysis	21238487

O60733	PLA2G6/85 kDa calcium independent phospholipase A2	sapiens (Human) Homo sapiens (Human)				x	x	Parkinson's Disease							21238487
P05231	Interleukin 6 (IL-6)	Homo sapiens (Human)			x	x	Anxiety	Up-regulated	18-65	M/F					23399050
P05231	Interleukin 6 (IL-6)	Homo sapiens (Human)			x	x	Depression	Up-regulated	18-65	M/F					23399050
P02741	C reactive protein (CRP)	Homo sapiens (Human)			x	x	Anxiety	Up-regulated	18-65	M/F					23399050
P02741	C reactive protein (CRP)	Homo sapiens (Human)			x	x	Depression	Up-regulated	18-65	M/F					23399050
P05112	IL-4	Homo sapiens (Human)	x			x	Schizophrenia								9300724
P01579	IFN-gamma	Homo sapiens (Human)	x			x	Schizophrenia								9300724
P22301	IL-10	Homo sapiens (Human)	x			x	Schizophrenia								9300724
P60568	IL-2	Homo sapiens (Human)	x			x	Schizophrenia								9300724
P05113	IL-5	Homo sapiens (Human)	x			x	Schizophrenia								9300724
P01375	TNF-alpha	Homo sapiens (Human)	x			x	Schizophrenia								9300724
P01374	TNF-beta/LT	Homo sapiens (Human)	x			x	Schizophrenia								9300724
Q8WXU2	Dyslexia susceptibility 1 candidate - dyx1c1	Homo sapiens (Human)				x	x	Dyslexia	Up-regulated				Southern Blotting, Immunohistochemical Study of Brain	non-proteomics	12954984
Q16552	IL-17	Homo sapiens (Human)		x			x	Autism		Mean 4	77M	collection in acid-citrate-dextrose Vacutainers	ELISA	non-proteomics	19800697
Q9NPF7	IL-23	Homo sapiens		x			x	Autism	Down-regulated	Mean 4	77M	collection in acid-citrate-	ELISA	non-proteomics	19800697

		(Human)									dextrose Vacutainers				
P20783	Neurotrophin-3 (NT-3)	Homo sapiens (Human)			x	x	Autism	Down-regulated			Blood drawn	ELISA, xMAP Luminex	non-proteomics	16289943
P05019	IGF1 Insulin-like growth factor I	Homo sapiens (Human)	x			x	Autism	Down-regulated	1-15	20M/5F		radio immunoassay	non-proteomics	16904022
P01344	IGF2 Insulin-like growth factor II	Homo sapiens (Human)	x			x	Autism		1-15	20M/5F		radio immunoassay	non-proteomics	16904022
P34130	Neurotrophin -4/5	Homo sapiens (Human)			x	x	Autism				Blood drawn	ELISA, xMAP Luminex	non-proteomics	16289943
Q9NPY3	Complement C1q	Homo sapiens (Human)		x		x	Autism	Up-regulated	4-6		Blood draw	LC-ESI-MS on TOF	proteomics	17189958
P02751	Fibronectin 1	Homo sapiens (Human)		x		x	Autism		4-6		Blood draw	LC-ESI-MS on TOF	Proteomics	17189958
P06727	Apolipoprotein A-IV	Homo sapiens (Human)		x		x	Autism	Up-regulated	4-6		Blood draw	LC-ESI-MS on TOF	Proteomics	17189958
P04114	Apolipoprotein B-100	Homo sapiens (Human)		x		x	Autism	Up-regulated	4-6		Blood draw	LC-ESI-MS on TOF	Proteomics	17189958
Q03591 P62258	Complement factor H - related protein	Homo sapiens (Human)		x		x	Autism		4-6		Blood draw	LC-ESI-MS on TOF	Proteomics	17189958
	Chain A, 14-3-3 Protein Epsilon	Mus musculus (Mouse)	x				Attention deficit hyperactivity disorder (ADHD)				Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
Q16555	Dihydropyrimidinase related protein-2 (DRP-2)	Mus musculus (Mouse)	x				Attention deficit hyperactivity disorder (ADHD)				Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
Q14195	Collapsin response mediator protein 4 (CRMP4)	Mus musculus (Mouse)	x				Attention deficit hyperactivity disorder (ADHD)				Dissection of brain and separation of cortex,	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR,	Proteomics and Transcriptomics	18457438

					striatum and midbrain on ice	western blot mass fingerprinting/tandem mass spectrometry		
P63104	14-3-3 Protein Zeta	Mus musculus (Mouse)	x	Attention deficit hyperactivity disorder (ADHD)	Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
P30086	Phosphatidylethanolamine binding protein	Mus musculus (Mouse)	x	Attention deficit hyperactivity disorder (ADHD)	Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
P49789	Fragile histidine triad protein (Fhit)	Mus musculus (Mouse)	x	Attention deficit hyperactivity disorder (ADHD)	Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
P11216	Brain glycogen phosphorylase	Mus musculus (Mouse)	x	Attention deficit hyperactivity disorder (ADHD)	Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
P18669	Phosphoglycerate mutase 1 (PGM1)	Mus musculus (Mouse)	x	Attention deficit hyperactivity disorder (ADHD)	Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
P60174	Triosephosphate isomerase (Tpi) 1 protein	Mus musculus (Mouse)	x	Attention deficit hyperactivity disorder (ADHD)	Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
P08559	Pyruvate dehydrogenase E1 alpha 1	Mus musculus	x	Attention deficit hyperactivity disorder (ADHD)	Dissection of brain and	Two-dimensional gel electrophoresis, Peptide	Proteomics and Transcriptomics	18457438

		(Mouse)			separation of cortex, striatum and midbrain on ice	mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot		
O00429	D100 (Dynamin 1)	Mus musculus (Mouse)	x	Attention deficit hyperactivity disorder (ADHD)	Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
Q9H115	N-ethylmaleimide sensitive fusion protein attachment protein (SNAP)-beta	Mus musculus (Mouse)	x	Attention deficit hyperactivity disorder (ADHD)	Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
P61764	Syntaxin binding protein 1	Mus musculus (Mouse)	x	Attention deficit hyperactivity disorder (ADHD)	Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
P22676	Calbindin 2 (Calretinin)	Mus musculus (Mouse)	x	Attention deficit hyperactivity disorder (ADHD)	Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
P49411	Tu translation elongation factor (EF-Tu)	Mus musculus (Mouse)	x	Attention deficit hyperactivity disorder (ADHD)	Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
P05388	Acidic ribosomal phosphoprotein PO	Mus musculus (Mouse)	x	Attention deficit hyperactivity disorder (ADHD)	Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438

Q99719	CDCrel-1AI	Mus musculus (Mouse)	x			Attention deficit hyperactivity disorder (ADHD)				Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
Q14568	Heat shock protein HSP 90-alpha	Mus musculus (Mouse)	x			Attention deficit hyperactivity disorder (ADHD)				Dissection of brain and separation of cortex, striatum and midbrain on ice	Two-dimensional gel electrophoresis, Peptide mass fingerprinting/tandem mass spectrometry, RT-PCR, western blot mass fingerprinting/tandem mass spectrometry	Proteomics and Transcriptomics	18457438
P09172	protein dopamine-b-hydroxylase	Homo sapiens (Human)		x		Attention deficit hyperactivity disorder (ADHD)	Down-regulated					non-proteomics	17187001
P0C0L5	complement C4B protein			x	x	Attention deficit hyperactivity disorder (ADHD)	Down-regulated	4-19	19M/4F	Blood collection	ELISA	non-proteomics	7665439
P30086	PEBP	Mus musculus (Mouse)	x			Attention deficit hyperactivity disorder (ADHD)					Electrophoresis 2D, RT-PCR, MALDI-TOF-MS	Proteomics and Transcriptomics	22231835
P35638	CHOP	Mus musculus (Mouse)	x			Attention deficit hyperactivity disorder (ADHD)					Electrophoresis 2D, RT-PCR, MALDI-TOF-MS	Proteomics and Transcriptomics	22231835
P30101	Grp58	Mus musculus (Mouse)	x			Attention deficit hyperactivity disorder (ADHD)						Proteomics	20109102

7.5 Annex 5: Band to band Kruskal-Wallis statistical analysis

	Kruskal-Wallis test	
9 kDa	Approximate P value	0,4968
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
9,5 kDa	Approximate P value	0,7916
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
10 kDa	Approximate P value	0,4639
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
11/12 kDa	Approximate P value	0,5661
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
13 kDa	Approximate P value	0,8285
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
16 kDa	Approximate P value	0,6619
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
19 kDa	Approximate P value	0,7668
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
22/23 kDa	Approximate P value	0,4671
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
25 kDa	Approximate P value	0,2214
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
27/28 kDa	Approximate P value	0,0843
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
29/30 kDa	Approximate P value	0,0019
	P value summary	**
	Do the medians vary signif. (P < 0.05)	Yes
31 kDa	Approximate P value	0,4639
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
33/34 kDa	Approximate P value	0,0885
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
35 kDa	Approximate P value	0,0028
	P value summary	**
	Do the medians vary signif. (P < 0.05)	Yes
37/38 kDa	Approximate P value	0,8424
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
43 kDa	Approximate P value	0,4968
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
46/47 kDa	Approximate P value	0,7994
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
54/56 kDa	Approximate P value	0,6525
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No

	Kruskal-Wallis test	
62/63 kDa	Approximate P value	0,7056
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
67/68 kDa	Approximate P value	0,1107
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
70/71 kDa	Approximate P value	0,4135
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
77/78 kDa	Approximate P value	0,8133
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
83 kDa	Approximate P value	0,5724
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
101/103 kDa	Approximate P value	0,1767
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
109 kDa	Approximate P value	0,2214
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
115/116 kDa	Approximate P value	0,5139
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
132 kDa	Approximate P value	0,5724
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
142 kDa	Approximate P value	0,5724
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
144 kDa	Approximate P value	0,2453
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
150 kDa	Approximate P value	0,4968
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
153-156 kDa	Approximate P value	0,0529
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
169/170 kDa	Approximate P value	0,7405
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
183 kDa	Approximate P value	0,4983
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
199/200 kDa	Approximate P value	0,0138
	P value summary	*
	Do the medians vary signif. (P < 0.05)	Yes
212 kDa	Approximate P value	0,1768
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No
238-240 kDa	Approximate P value	0,7474
	P value summary	ns
	Do the medians vary signif. (P < 0.05)	No